CHARACTERIZATION OF LRRTM AND NGR GENE FAMILIES:

EXPRESSION AND FUNCTIONS

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals **(I-IV)**:

I **Laurén J**., Airaksinen M.S., Saarma M., and Timmusk T.: A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system. *Genomics*, 2003, 81(4): 411-421

II **Laurén J**., Airaksinen M.S., Saarma M., and Timmusk T.: Two Novel Mammalian Nogo Receptor Homologs Differentially Expressed in the Central and Peripheral Nervous System. *Molecular and Cellular Neuroscience*, 2003, 24(3): 581-594

III **Laurén J**., Hu F., Chin J. Liao J., Airaksinen M.S., and Strittmatter S.M.: Characterization of Myelin Ligand Complexes with Neuronal Nogo-66 Receptor Family Members. *Journal of Biological Chemistry*, 2007, 282(8): 5715-5725

IV Francks C., Maegawa S.*****, **Laurén J.***, Abrahams B., Velayos-Baeza A., Medland S.E., Colella S., Groszer M., McAuley E.Z., Caffrey T.M, Timmusk T., Pruunsild P., Koppel I., Lind P.A., Matsumoto-Itaba N., Nicod J., Xiong L., Joober R., Enard W., Krinsky B., Nanba E., Richardson A.J., Riley B.P, Martin N.G., Strittmatter S.M., Möller H-J., Rujescu D., Clair D., Muglia P., Roos J.L., Fisher S.E., Wade-Martins R., Rouleau G.A., Stein J.F., Karayiorgou M., Geschwind D.H., Ragoussis J., Kendler K.S., Airaksinen M.S., Oshimura M., DeLisi L.E., and Monaco A.P.: LRRTM1 on chromosome 2p12 is a maternally suppressed gene that is associated paternally with handedness and schizophrenia. *Molecular Psychiatry, 2007 (in press)*

*****Equal contribution

Also previously unpublished results are presented.

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SELECTED ABBREVIATIONS

cDNA, complementary DNA
CNS, central nervous system
DIV, days *in vitro*
DRG, dorsal root ganglion
E, embryonic day
ER, endoplasmic reticulum
EST, expressed sequence tag
GPI, glycosylphosphatidylinositol
HGP, Human Genome Project
IHGSC, international Human Genome Sequencing Consortium
kb, kilo base pairs
LRR, leucine-rich repeat
LRRTM, leucine-rich repeat transmembrane neuronal
MAG, myelin-associated glycoprotein
NCBI, National Center for Biotechnology Information
NGR, Nogo-66 Receptor
NGRL, NGR-like
OMgp, oligodendrocyte myelin glycoprotein
ORF, open reading frame
P, postnatal day
RHD, reticulon homology domain
RTN, reticulon
TM, transmembrane
UTR, untranslated region
SNP, single nucleotide polymorphism

ABSTRACT

Some leucine-rich repeat (LRR) -containing membrane proteins are known regulators of neuronal growth and synapse formation. In this work I characterize two gene families encoding neuronal LRR membrane proteins, namely the LRRTM (leucine-rich repeat, transmembrane neuronal) and NGR (Nogo-66 receptor) families.

I studied LRRTM and NGR family member's mRNA tissue distribution by RT-PCR and by *in situ* hybridization. Subcellular localization of LRRTM1 protein was studied in neurons and in non-neuronal cells. I discovered that LRRTM and NGR family mRNAs are predominantly expressed in the nervous system, and that each gene possesses a specific expression pattern. I also established that LRRTM and NGR family mRNAs are expressed by neurons, and not by glial cells. Within neurons, LRRTM1 protein is not transported to the plasma membrane; rather it localizes to endoplasmic reticulum.

Nogo-A (RTN4), MAG, and OMgp are myelin-associated proteins that bind to NgR1 to limit axonal regeneration after central nervous system injury. To better understand the functions of NgR2 and NgR3, and to explore the possible redundancy in the signaling of myelin inhibitors of neurite growth, I mapped the interactions between NgR family and the known and candidate NgR1 ligands. I identified high-affinity interactions between RTN2-66, RTN3-66 and NgR1. I also demonstrate that Rtn3 mRNA is expressed in the same glial cell population of mouse spinal cord white matter as Nogo-A mRNA, and thus it could have a role in myelin inhibition of axonal growth.

To understand how NgR1 interacts with multiple structurally divergent ligands, I aimed first to map in more detail the nature of Nogo-A:NgR1 interactions, and then to systematically map the binding sites of multiple myelin ligands in NgR1 by using a library of NgR1 expression constructs encoding proteins with one or multiple surface residues mutated to alanine. My analysis of the Nogo-A:NgR1 -interactions revealed a novel interaction site between the proteins, suggesting a trivalent Nogo-A:NgR1-interaction. Our analysis also defined a central binding region on the concave side of NgR1's LRR domain that is required for the binding of all known ligands, and a surrounding region critical for binding MAG and OMgp.

To better understand the biological role of LRRTMs, I generated Lrrtm1 and Lrrtm3 knock out mice. I show here that reporter genes expressed from the targeted loci can be used for maping the neuronal connections of Lrrtm1 and Lrrtm3 expressing neurons in finer detail.

With regard to LRRTM1's role in humans, we found a strong association between a 70 kb-spanning haplotype in the proposed promoter region of LRRTM1 gene and two possibly related phenotypes: left-handedness and schizophrenia. Interestingly, the responsible haplotype was linked to phenotypic variability only when paternally inherited.

In summary, I identified two families of neuronal receptor-like proteins, and mapped their expression and certain protein-protein interactions. The identification of a central binding region in NgR1 shared by multiple ligands may facilitate the design and development of small molecule therapeutics blocking binding of all NgR1 ligands. Additionally, the genetic association data suggests that allelic variation upstream of LRRTM1 may play a role in the development of left-right brain asymmetry in humans. Lrrtm1 and Lrrtm3 knock out mice developed as a part of this study will likely be useful for schizophrenia and Alzheimer's disease research.

1 REVIEW OF THE LITERATURE

1.1 Identifying protein-coding genes in the post-genomic era

In 1977 Frederick Sanger introduced a method to sequence DNA, known currently as chain termination method or the Sanger method (Sanger *et al.* 1977), which was to become the method used in sequencing of the human genome. The International Human Genome Project (HGP) was launched in 1990 with the goal of obtaining a highly accurate sequence of the vast majority of the euchromatic portion of the human genome.

The results of initial sequencing and analysis of the human genome were published in February 2001 by the International Human Genome Sequencing Consortium (IHGSC) (IHGSC 2001), and by Celera Genomics, Inc. (Venter *et al.* 2001). Both of these drafts lacked ~10% of the euchromatic sequence and ~30% of the genomic sequence as a whole. The authors of these studies suggested that the human genome harbors 30 000 – 40 000 (IHGSC 2001) or 24 000 – 40 000 (Venter *et al.* 2001) protein-coding genes. However, in October 2004 a more complete analysis of human genome, based on sequences covering ~99 % of the euchromatic sequence, led IHGCS to refine its estimate to 20 000 – 25 000 protein-coding genes (IHGSC 2004). Currently (build August 2006, database version 42.36d) the Ensembl human genome database (Hubbard *et al.* 2007) contains 21 774 protein-coding genes. The CCDS (consensus coding sequence) database contains protein-coding genes that different organizations involved in gene annotation agree to a single amino acid level. These CCDS contributors are European Bioinformatics Institute (EBI), National Center for Biotechnology Information (NCBI), Wellcome Trust Sanger Institute (WTSI), and University of California, Santa Cruz (UCSC)). In early 2007 CCDS contained only 13 132 genes (CCDS 2007).

The above mentioned numbers for protein-coding genes illustrate the iterative process of human genome analysis. The variation of the protein-coding gene number estimates, although converging by nature, also underscores the need for non-automatic curation of gene annotation and experimental verification of the proposed genes, and thus warranted our efforts that led to the cloning and characterization of LRRTM (leucine-rich repeat transmembrane neuronal) and NGRL (Nogo-66 receptor like) gene families.

The very recent changes in gene number estimates (*e.g.* March, 2006 build of Ensembl human genome database contained 21 561 protein-coding genes vs. the current 21 774 genes, as stated above) illustrate that the efforts to exhaustively catalogue the protein-coding genes in the human genome are still needed and ongoing.

In the recent years, efforts to identify protein-coding genes have been based on the following methods (Brent 2005):

- Analysis of expressed sequence tag (EST) cDNA clones. This historically successful approach provides direct experimental evidence for mRNA transcripts (Boguski *et al.* 1993). However, it is limited by the fact that as more and more transcripts are already known, it has became increasingly unlikely to identify novel transcripts perhaps expressed only in a small sub-set of cells in an organism. On Jan. 12th, 2007 NCBI's dbEST-database contained ~8x10^{6} EST sequences derived from human cDNA libraries.

- Genomic sequence database searches for homologous sequences. This enables identification of genes homologous to known genes. TBLASTN search tool, which performs conceptual translation of the searched nucleotide databases, is commonly used (Gish and States 1993). Additionally, using homology searches orthologous genes in other organisms can be identified as well.

- Aligning genomic sequences from multiple organisms. As the protein-

coding regions are more conserved than non-coding regions of the genome, the analysis of sequence conservation between species allows identification of protein-coding genes. This approach is, however complicated by non-protein-coding functional elements of the genome, which also display considerable or even extreme sequence conservation between species (Bejerano *et al.* 2004).

- Identifying genes based on their sequence composition. An example of computer programs using this approach is GenScan (Burge and Karlin 1997). GenScan identifies putative genes based on their characteristic transcriptional, translational and splicing signals, as well as typical features of exons, introns and intergenic regions.

These methods, plus manual annotation, are usually used in combination to come up with the best predictions (Brent 2005). However, the predicted gene structures should be experimentally verified (Brent 2005).

1.2 The *zeitgeist* of '*omics*' *vs.* bottom-up approaches in biology

The challenges of genome analysis are even significantly larger in areas other than identification of protein-coding genes. Since the discovery of non-protein coding transcribed DNA, the very definition of gene has been under debate (Pearson 2006). Identification of all genes satisfying the current suggested all-encompassing definition of a gene – a locatable region of genomic sequence corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions and/or other functional sequence regions – provides an even greater challenge (Pearson 2006). However, efforts to identify all functional non-protein-coding transcripts, as well as other regulatory elements in the human genome are ongoing (ENCODE 2004).

Sequencing the human genome arguably paved the way for other large-scale projects in biology. Hence, the '-ome' and '-omics' - suffixes in *'genome' and 'genomics'* were adapted into a short-hand notations *e.g.* for proteome and epigenome, the entity of all the proteins and all DNA modifications, respectively.

A notable example of current significant large-scale projects in biology is the HapMap project, which has already elucidated the common single nucleotide polymorphisms (SNPs) in the certain human populations (HapMap Consortium 2005).

The databases containing the results of the human and mouse genome projects and the HapMap database were used in this thesis work. The availability of genomic sequences was instrumental in gene identification, and the availability of HapMap data facilitated the study on the role of LRRTM1 locus polymorphism in humans. Thus, results from large-scale projects can greatly benefit numerous smaller-scale projects that aim to understand biology by working the way 'from bottom to up', from individual details towards overall understanding of the system's functions.

In contrast, other large-scale projects seem likely to render certain current scientific practices either obsolete or forces them to define a new niche that provides *raison d'être*. A recent example of this development is the Allen Brain Atlas, which provides a well-accessible cellular-resolution, genome-wide map of gene expression in the mouse brain (Lein *et al.* 2007). Currently, this digital atlas contains ~$1x10^6$ images and provides mouse brain expression data obtained by *in situ hybridization* with probes targeting ~20 000 genes. The data in Allen Brain Atlas is consistent with what I have reported for LRRTM and NGR gene families (**I, II,** and unpublished data). The Allen Brain Atlas reduces the need to perform *in situ* hybridizations to more specific situations, *e.g.* as was also done in this work when i) I assessed the developmental expression of Lrrtm1 in mouse brain structures relevant for Schizophrenia pathogenesis **(IV)**, and ii) when I assessed the expression of reticulon (RTN) family mRNA transcript splice variants containing the RTN homology domain (RHD) in adult mouse spinal cord white matter **(III)**.

Arguably, certain types of projects are less amenable to automation and offer lesser economies of scale than others. The productivity of work at dedicated genome sequencing centers rose sharply during the human genome project (IHGSC 2001). The proposed project to generate a knock out mouse model for every protein-coding gene, on the other hand, has had only moderate success, and the projected goal of the project for the years 2006 – 2010 is to generate in total 920 new knock out mouse lines. (The International Mouse Knockout Consorti um 2007). This represents only 4% of protein-coding genes in the mouse genome. Because of the expected limited output, the project participants have agreed to prioritize which genes to knock out first. Also, the possibility to create null, hypomorphic, or conditional knock outs seems likely to drive demand for custom-designed knock out mice for years to come. The current fragmentary model to generate knock out mice may even remain as the mainstream method. We generated Lrrtm1 and Lrrtm3 knock out mice lines during this thesis work. These genes have not been currently targeted by the large scale mouse knock out projects.

In summary, based on the examples given above, the large-scale 'omic' projects have greatly benefited the biological inquiry, but 'from bottom to up', or hypothesis-driven approaches to biological inquiry seem likely to prevail for the time being. This thesis illustrates well the challenges biological research faces when trying to find a balance between gene-driven and genome-driven biological paradigms. Clearly the ongoing large-scale data-driven projects need to be supported with more advanced data storage and annotation systems than the current format of printed scientific publications.

1.3 How to elucidate what a gene does: from gene to phenotype or *vice versa*?

Traditionally functions of genes have been studied by means of "forward genetics" (from phenotype to gene). In the field of medical genetics this positional cloning of gene defects underlying hereditary phenotypes was greatly accelerated by advanced maps of the human genome, and similar approaches have also been used to map gene variants underlying mouse phenotypes (Takahashi *et al.* 1994). In mouse and other model organisms these efforts were also accelerated by the possibility to create random mutations. Analogously, forward genetic approaches are used to manipulate the DNA content of individual cells, followed by identification of the cells displaying the desired phenotype. This is then, again, followed by the identification of DNA changes that induced the phenotype (Stark and Gudkov 1999). Examples of cell-based "forward genetics" approaches are various two-hybrid screenings, and expression cloning strategies to identify cell surface receptors (see *e.g.* (Fournier *et al.* 2001)).

The genome projects and other simultaneous efforts to analyze genomic sequences led to the identification of a great number of genes without known functions (IHGSC 2001). An example is my discovery of LRRTM and NGRL gene families. A plausible approach to study the functions of these functionally orphan genes would combine bioinformatics, molecular and cell biology methods, and analysis of genetically engineered model organisms. Since no information about gene's function is available in the beginning of the study, this approach necessarily includes a 'leap of faith' that something biologically important will be discovered.

This dichotomist view to elucidate gene's functions has evolved; reverse and forward genetic approaches can be considered as complementary. As the positional cloning efforts to identify gene variants predisposing to multifactorial diseases have largely failed (Hirschhorn and Daly 2005), the reverse genetics approach could reveal important insights into the functions of the candidate genes and hence strengthen or weaken the evidence linking variation in a certain gene to a certain phenotypic trait. This interdisciplinary approach was used in this thesis work when analyzing the role of LRRTM1 gene in the determination of handedness and schizophrenia susceptibility **(IV)**.

1.4 The leucine-rich repeat protein superfamily

The LRRTM and NGRL gene families described in this work encode proteins with leucine-rich repeat (LRR) domains as a dominant feature.

1.4.1 Structure of the LRR domain

Leucine-rich repeats consist of 20–29 amino acid long repetitive sequences with a variable segment and a more conserved segment, defined by a consensus sequence LxxLxLxxNxL, or LxxLxLxxCxxL, in which "L" is Val, Leu, or Ile, "N" is Asn, Thr, Ser, or Cys, and "C" is Cys or Ser (Kobe and Kajava 2001; Enkhbayar *et al.* 2004). LRRs are present in a tandem fashion, varying in known cases from 2 to 45 repeats (Enkhbayar *et al.* 2004). An uninterrupted set of LRRs is referred as a LRR domain. Based on variation in the variable region, LRRs are classified into seven subfamilies, the most common being named as a "typical" subfamily (Enkhbayar *et al.* 2004). The LRR domain in LRRTM and NGRL family members belongs to this "typical" subfamily. Typically in LRR proteins destined for the extracellular/secretory pathway, the amino- and carboxyl-terminal LRRs are flanked by cysteine-rich flanking domains (LRRNT and LRRCT, respectively). The typically four cysteines in flanking domains form two disulphide bond pairs. By shielding the otherwise exposed hydrophobic core at the ends of the LRRs, these capping domains serve essential functions in the folding of the LRR domain. These capping modules are usually classified as being a part of the LRR domain (Kobe and Kajava 2001).

Individual LRRs correspond to basic structural units in the LRR domain. Each unit consists of a β strand and adjacent loop regions (which corresponds to the conserved segment in the consensus sequence), and an α helix or less orderly loop structure (which corresponds to the more variable segment in the consensus sequence). β strands are aligned in a parallel fashion on the concave side of the LRR domain whereas α helix or loop structures are located on the convex side. The LRR domain forms a non-globular, more or less curved structure **(Figure 1)**. In addition to curving around a single axis (arc or also called as banana, or horseshoe shape) some LRRs show some degree of twist around another axis. The extended shape of LRR domain offers opportunities for molecular interactions (Kobe and Kajava 2001).

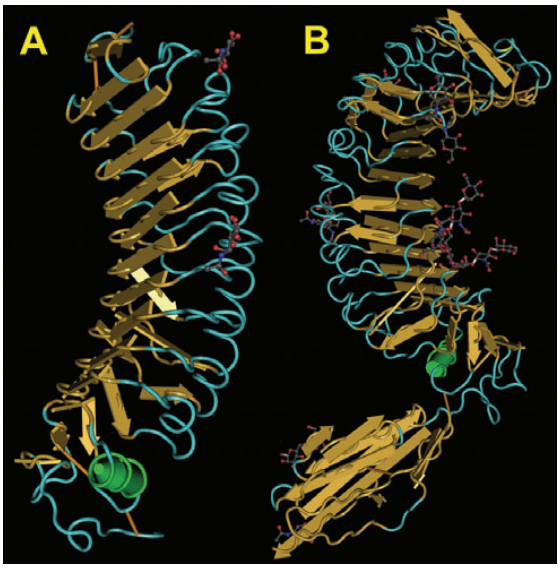

***Figure 1*. Crystal structure of NgR1 (A) and LINGO-1 (B) Ectodomains**. *β-sheets are visualized with yellow arrows, and α-helical structures are shown in green. Observed carbohydrate moieties are also shown. For NgR1 the structure contains only LRR domain, whereas the structure of Lingo-1 encompasses LRR and IgG-like domains. The illustrations were done using Cn3D 4.1 software (NCBI), and they are based on the atomic coordinate files deposited to NCBI Structure database: 1P8T (human NgR1 (Barton et al. 2003)) and 2ID5 (human LINGO-1 (Mosyak et al. 2006)). NgR1 and LINGO1 are not in same scale.*

Biochemical experiments have demonstrated that several LRR proteins show homophilic adhesion or heterophilic adhesion among family members (Fournier *et al.* 2002; Kuja-Panula *et al.* 2003; Karaulanov *et al.* 2006); these findings are supported and possibly mechanistically explained by the observed packing of LRR protein monomers into crystals used for determining the structure of NgR1 and LINGO-1 (Barton *et al.* 2003; Mosyak *et al.* 2006).

1.4.2 Prevalence of the LRR domains in the proteome

Genes encoding LRR domain-containing proteins are present in eukaryotes and prokaryotes. InterPro (Mulder *et al.* 2007) database contains 14 functionally uncharacterized LRR proteins from *Archaea* (*e.g.* proteins with UniProt database accession numbers: Q2NHF7, Q648Z4 and Q8TNI4) Interestingly, in plants, LRR-containing receptor-like kinases (RLKs) compose a large family of proteins (216 members in *A. thaliana*). Each RLK encompasses an extracellular LRR domain, a transmembrane segment, and a cytoplasmic serine/threonine protein kinase domain. RLKs have diverse roles in transduction of extracellular signals to cells (Dievart and Clark 2004). In jawless vertebrates the variable lymphocyte receptors consist of LRRs. Analogously to immunoglobulin-type antigen receptors in other vertebrates, the somatic diversification of these lymphocyte receptors occurs by random selection and assembly of LRRs (Alder *et al.* 2005).

As of January 2007 InterPro database contained 571 entries (genes) encoding proteins with LRR domain(s). As InterPro database of protein families, domains and functional sites combines data from multiple sources, it provides the current best estimate for the prevalence of this domain. Thus, ~2% of human proteins have LRR domain. However, it should be noted that currently InterPro database does not exhaustively cover the entire human transcriptome (Mulder *et al.* 2007). Therefore, although the exact number of LRR-encoding proteins is currently unclear, it is likely close to 571, making the LRR domain undoubtedly a very commonly occurring protein domain in human proteome.

Gene/ gene family	Expression pattern	Subcellular protein localization	Protein structure	Biological functions	Selected reference
Toll-like receptor (TLR) family (11 members)	immune cells	plasma membrane, endosomes	LRR domain, TM segment, intracellular part	Innate immunity (recognition of microbial components)	(Akira *et al.* 2006)
Polycystin1	widely expressed	plasma membrane, endoplasmic reticulum	large extracellular region with several domains (incl. LRR), 11 membrane-spanning segments, cytoplasmic G protein binding site	Forms a Ca-ion channel; other functions; mutations cause autosomal dominant polycystic kidney disease (ADPKD).	(Ong and Harris 2005)
Leucine-rich repeat-containing G protein coupled receptors (GPCR), (LGRs) (8 members)	widely expressed; differs between family members	plasma membrane	extracellular LRR domain - containing G protein coupled receptors (GPCRs)	Glycoprotein hormone receptors: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) receptors, LGR4-8 orphan receptors	(Hsu *et al.* 2000)
Small leucine-rich repeat proteoglycans (SLRPs) (at least nine members)	differs between family members	extracellular matrix	LRR domain, attached glycosaminoglycan moieties	Regulate cell growth, adhesion, and migration	(Hocking *et al.* 1998)
Scribble	widely expressed	cytoplasm and nucleus	LRR domain, multiple C-terminal PDZ domains	Regulates cell polarity and differentiation; potential tumor suppressor gene	(Navarro *et al.* 2005)
Platelet glygoprotein Ib α	megakaryo-blasts, platelets	plasma membrane	LRR domain, TM segment, intracellular part	Platelet adhesion	(Huizinga *et al.* 2002)
Slit family (3 members)	predominantly neuronal	extracellular matrix	complex domain architecture; N-terminal LRR domain is critical for receptor binding	Signal via Robo receptors to guide axonal growth and angiogenesis	(Wong *et al.* 2002)
LRRK family (2 members)	predominantly neuronal	cytoplasmic	LRR, GTPase, COR, kinase, and WD40 domains	LRRK2 is the most commonly mutated gene in autosomal dominant Parkinson's disease	(Mata *et al.* 2006)
LGI family (4 members)	predominantly neuronal	secreted	LRR domain and a EPTP repeat region	Enhances AMPA receptor-mediated synaptic transmission; mutated in autosomal dominant lateral temporal epilepsy (ADLTE)	(Fukata *et al.* 2006)
Neuronal type I TM or GPI-anchored proteins	predominantly neuronal	plasma membrane, secretory pathway	see **Table 2**	see **Table 2**	see **Table 2**

Table 1. Examples of human proteins with LRR domain. *LRRK, leucine-rich repeat kinase; COR, C-terminal of Roc; EPTP*

Selected examples of human LRR proteins are shown in **Table 1**. Examples were selected to illustrate the diverse roles LRR domain containing proteins serve. The table also exemplifies that LRRs are present in secreted proteins, in proteins spanning the plasma membrane or other cellular lipid bilayers, and in intracellular proteins. Not surprisingly, many genes encoding LRR proteins are mutated in known human diseases (for review, see (Matsushima et al. 2005)).

Bases on sequence homology, LRRTM and NGR families belong to the most populated "typical" subfamily of LRR proteins. In humans, according to the InterPro database, this subfamily has 257 annotated members.

1.4.3 On the evolution of genes encoding proteins with signal peptide and LRR domain

The family of genes encoding signal-peptide containing LRR proteins seems to have expanded dramatically since the divergence of vertebrate and arthropod lineages: I identified 12 *Drosophila melanogaster* genes encoding LRR domain and signal peptide from UniprotKB database (Uniprot Consortium 2007), whereas 100 human entries satisfied the same criteria. These numbers are, however, not absolute, as annotation of signal peptides for some entries (for those derived from trEMBL database) depends on whether the original data submitter annotated that feature or not (Dr. Nicky Mulder, European Bioinformatics Institute, personal communication). Nonetheless, assuming that the fruit fly genome is at least as well annotated as that of humans, these numbers demonstrate the rapid expansion of LRR membrane protein family during vertebrate evolution. Literature analysis based on the identified fruit fly proteins shows that many of these proteins, recognized by database and additional literature searches, possess a transmembrane (TM) segment, in addition to LRR domain and signal peptide, and possibly an IgG-like domain. Well characterized examples are capricious and tartan (signal peptide, 14 LRRs, TM segment, short intracellular region) (Milan *et al.* 2001) and kekkon family members (6 genes encoding proteins with seven LRRs, IgG-like domain, TM segment and a short intracellular region) (MacLaren *et al.* 2004). Capricious and tartan have a role in regulating cell adhesion (Milan *et al.* 2001), and kekkon-1 negatively regulates the activity of the drosophila EGF (epidermal growth factor) receptor (Ghiglione *et al.* 1999).

Given the rapid expansion of LRR membrane proteins during vertebrate evolution, invertebrate orthologs for specific vertebrate LRR membrane proteins can generally not be determined unambiguously using sequence homology analysis. In contrast, due to the sequence conservation of LRR proteins during vertebrate evolution (*e.g.* (Kuja-Panula *et al.* 2003), **I, II**), the paralogs belonging to a particular family of vertebrate LRR proteins can usually be determined.

1.5 Neuronal LRR membrane proteins

Literature analysis of vertebrate genes encoding proteins with signal peptide, LRR domain, and TM segment/GPI anchor shows that in most known cases, these genes are highly expressed in the nervous system. This prominently neuronal expression pattern has been noticed earlier (Chen *et al.* 2006). By analyzing protein databanks in conjunction with exhaustive literature searches I identified 41 human genes satisfying the following criteria: i) has a published reference verifying that the gene or a closely related family member is neuronally expressed (i.e. all family members were included if at least one member of the family is known to be predominantly neuronally expressed); ii) presumably encodes a protein possessing a signal peptide, LRR domain, a single TM segment or GPI-anchor modification site, and optionally some additional domains. These 41 gene products are referred here as *"neuronal LRR membrane proteins"*, and they are listed in **Table 2**. For some genes encoding type I TM LRR proteins expression pattern is not established, and several genes are not characterized elsewhere than in bioinformatics databases. These genes are excluded from **Table 2**. Some genes

encoding type I TM LRR proteins are clearly not neuronally expressed or enriched (most importantly toll-like receptor family (Akira *et al.* 2006)).

Notably, the majority of LRR proteins do not contain transmembrane segments or GPI-anchor modification sequences; they are either secreted or intracellular proteins. In these LRR protein subfamilies, a great diversity of domains occurs in tandem with LRR domain.

1.5.1 Structural features of neuronal LRR membrane proteins

Neuronal LRR membrane proteins typically contain ~10 LRRs (from 5 to 15). Other domains commonly occurring in concert with LRR domains in neuronal LRR membrane proteins are immunoglobulin (IgG) -like domain and fibronectin (Fn) type III repeat: IgG-like domain is present in 20 and Fn type III repeat in 13 out of these 41 proteins. The order of domains in the polypeptide chain is defined: if other domains than LRR-domain are present, then IgG-like domain(s) lie carboxyl-terminal to the LRR-domain, and Fn type III repeat lies carboxyl-terminal to the IgG-like domain(s). The role of IgG-like domains and Fn type III repeat domains occurring in these proteins has not been studied extensively. In the case of NGL-1, the IgG-like domain was found not to have a role in Netrin-G1 binding (Lin *et al.* 2003). In LINGO-1 the IgG-like domain was noted to cause a sharp ~90º angle between the LRR and IgG-like domains, and thereby placing the IgG-like domain in close apposition with the convex side of the LRR domain (Mosyak *et al.* 2006). This induced turn in the LINGO-1 polypeptide chain orientation could be critical for presenting the associated LRR domain in a correct orientation in relation to the plasma membrane.

***Table 2. Genes encoding neuronal LRR membrane proteins described in the literature. LRRTM**, leucine-rich repeat transmembrane neuronal; **SLITRK**, Slit and Trk-like; **Amigo**, amphoterin-induced gene and ORF; **Alivin-1**, activity-dependent leucine-rich repeat and Ig superfamily survival-related protein; **DEGA**, differentially expressed in human gastric adenocarcinoma; **NGL**, netrin-G1 ligand; **LRRC**, leucine-rich repeat containing; **LINGO**, LRR and Ig domain-containing, nogo receptor-interacting protein; **LERN1**, leucine-rich repeat neuronal protein 1; **LRRN6A**, leucine-rich repeat neuronal protein 6; **FLRT**, fibronectin-leucine-rich transmembrane; **NLRR**, neuronal leucine-rich repeat protein; **LRIG**, leucine-rich repeats and immunoglobulin-like domains protein; **LIG-1**, leucine-rich repeats and immunoglobulin-like domains 1; **SALM**, synaptic adhesion-like molecule; **Lrfn**, Leucine-rich repeat and fibronectin type III domain-containing; **OMgp**, oligodendrocyte myelin glycoprotein; **NgR**, Nogo-66 receptor. S/T, serine/threonine rich domain.*

Family name	Family members	Subcellular protein localization	Protein structure						Biological function(s)	Reference(s)
			LRRs	IgG-like	Fn III	other	TM/ GPI	Intracellular		
LRRTMs	LRRTM1 LRRTM2 LRRTM3 LRRTM4	ER, other?	10	-	-	-	TM	yes	See *Results* -chapter	**(I)**, (Haines and Rigby 2007)
SLITRKs	SLITRK1 SLITRK2 SLITRK3 SLITRK4 SLITRK5 SLITRK6	Trans-golgi network, plasma membrane	11	-	-	-	TM	yes	Overexpression in neurons affects neurite outgrowth *in vitro*. Rare variants of SLITRK1 possibly associated with Tourette's syndrome.	(Aruga and Mikoshiba 2003; Aruga *et al.* 2003; Abelson *et al.* 2005)
Amigos	AMIGO1 AMIGO2/ Alivin-1/ DEGA AMIGO3	likely plasma membrane	6	1	-	-	TM	yes	Promote neurite outgrowth and fasciculation *in vitro*; Amigo2/alivin-1 inhibits neuronal apoptosis *in vitro*.	(Kuja-Panula *et al.* 2003; Ono *et al.* 2003; Rabenau *et al.* 2004)
NGLs	NGL-1 NGL-2/LRRC4 NGL-3	plasma membrane	9	1	-	-	TM	yes	NGL-1 binds to Netrin G1 and attracts thalamic neurons. NGLs promote synapse formation.	(Lin *et al.* 2003; Zhang *et al.* 2005; Kim *et al.* 2006)
LINGOs	LINGO-1/ LERN1/ LRRN6A LINGO-2 LINGO-3	plasma membrane	12	1	-	-	TM	yes	Negative regulator of myelination *in vivo*. Role in regeneration *in vivo*. Co-receptor for NgR1.	(Carim-Todd *et al.* 2003; Mi *et al.* 2004; Mi *et al.* 2005; Ji *et al.* 2006; Lee *et al.* 2007)
FLRTs	FLRT-1 FLRT-2 FLRT-3	plasma membrane	10	-	1	-	TM	yes	Overexpression of FLRT-3 promotes neurite outgrowth *in vitro*. *Xenopus Laevis* FLRT-3 interacts with FGFRs. FLRTs interact homophilically	(Lacy *et al.* 1999; Bottcher *et al.* 2004; Robinson *et al.* 2004; Tsuji *et al.* 2004; Karaulanov *et al.* 2006)
NLRRs	NLRR-1 NLRR-2/GAC1 NLRR-3 NLRR-5	plasma membrane	12	1	1	-	TM	yes	NLRR-3 facilitates EGF internalization and signaling.	(Taguchi *et al.* 1996; Fukamachi *et al.* 2002; Hamano *et al.* 2004)
	NLRR-4	n.d.	11	-	1	-	TM	yes	Not parologous to NLRR1-3. NLRR-4 knock out mice have memory deficits.	(Bando *et al.* 2005)
LRIGs	LRIG-1/ LIG-1 LRIG-2	plasma membrane	15	3	-	-	TM	yes	LRIG-1 is expressed predominantly in glial cells. LRIG-1 enhances ubiquitylation and degradation of EGFR *in vitro*. LRIG-1 null mice develop skin disorder reminiscent of psoriasis.	(Suzuki *et al.* 1996; Suzuki *et al.* 2002; Nilsson *et al.* 2003; Gur *et al.* 2004; Laederich *et al.* 2004)
SALMs	SALM1 SALM2 SALM3 SALM4 SALM5 (also called as *Lrfn*s)	plasma membrane	6	1	1	-	TM	yes	Regulate multiple aspects of neuronal differentiation, including neurite outgrowth, spine formation, and assembly of excitatory synapse apparatus.	(Ko *et al.* 2006; Wang *et al.* 2006) (Morimura *et al.* 2006)
	Lib/LRRC15	plasma membrane	15	-	-	-	TM	yes	Expression induced by β-amyloid in rat astrocytes *in vitro*.	(Satoh *et al.* 2002; Satoh *et al.* 2005)
	Pal	ER, secretory pathway; not on the plasma membrane	5	1	1	-	TM	yes	Retina-specific expression.	(Gomi *et al.* 2000)
	Nyctalopin	plasma membrane	11	-	-	-	GPI	-	Expressed in the retina, brain and in some other organs. Mutated in X-linked complete congenital stationary night blindness	(Bech-Hansen et al. 2000; Pusch et al. 2000; Pesch et al. 2003; Zeitz et al. 2003)
	OMgp	plasma membrane	8	-	-	S/T	GPI	-	Binds Ngr1 and MAG. Inhibits neurite outgrowth *in vitro* and *in vivo*.	(Wang et al. 2002; Vourc'h and Andres 2004; Huang et al. 2005)
NgRs	NgR1 NgR2 NgR3	plasma membrane	8	-	-	-	GPI	-	NgR1 and likely NgR2 mediate 'myelin inhibition of neurite growth'	**(II)**, (Barton *et al.* 2003; Pignot *et al.* 2003)

Only OMgp (oligodendrocyte myelin glycoprotein) contains some additional extracellular elements not discussed above: it contains a unique serine-threonine rich region between the LRR domain and GPI-anchor modification site. It is unclear whether this segment folds and forms a globular protein domain structure or it provides a less ordered linker sequence between the LRR domain and the TM segment.

OMgp, NgR family members (3 genes) and nyctalopin are GPI-anchored proteins. Other neuronal LRR membrane proteins contain a relatively small (~50-100 amino acid long) intracellular part following the TM segment. None of these 36 neuronal type I transmembrane LRR proteins contains an intracellular part with enzymatic activity, and the possible downstream signaling events triggered by these proteins are unclear at the molecular level. Several type I transmembrane LRR proteins contain a carboxyl-terminal sequence that could serve as a binding site for PDZ-proteins (named after three first identified proteins with PDZ-domain: PSD-95, Discs-large, and ZO-1). PDZ-domain containing proteins are involved in diverse physiological processes. Importantly many of them serve scaffolding functions in synapses (Hung and Sheng 2002; Kim and Sheng 2004). SALM1, SALM2, and NGL-2 associate with PSD-95, as well as with other PDZ-domain containing proteins present in the synapse (Kim *et al.* 2006; Ko *et al.* 2006; Wang *et al.* 2006).

1.5.2 Emerging common themes in neuronal LRR membrane protein biology

In vitro experiments have established that most neuronal LRR membrane proteins are transported to the plasma membrane. Notably, Pal is known to localize predominantly along the secretory pathway (Gomi *et al.* 2000), and my observations suggest the same for LRRTM1 **(IV)**. Subcellular localization of neuronal LRR membrane proteins has been typically assessed by overexpresssing the gene-of-interest in cell lines. No studies addressing subcellular localization of any endogenous neuronal LRR membrane protein using well characterized antibody (demonstrated by showing lack of signal in knock out tissue sections) have been performed. Even by using less stringently characterized antibodies, only NgR1 has been shown to be localized at least partially to the plasma membrane (Wang *et al.* 2002). The PDZ-domain binding motif, which is present in several members of the neuronal LRR membrane protein family, seems to play an essential role in regulating their protein-protein-interactions at least in some cases, and could thus control their intracellular localization as well (Kim *et al.* 2006; Wang *et al.* 2006).

Several neuronal LRR membrane proteins have been suggested to be involved in regulating neurite outgrowth (at least Slitrks, Amigos, FLRTs, SALMs, NgR1, NgR2, OMgp, NGL-1; references in **Table 2**) either when overexpressed in neurons or presented as a soluble or surface-bound exogenous proteins. This is consistent with the LRR domain's role in protein-protein–interactions; it could therefore affect the adhesion of neurons to substrates. Alternatively, intracellularly localized pools of proteins could regulate the transport of other proteins needed for neurite outgrowth. However, it should be noted that experiments measuring neurite outgrowth are likely to be among the first *in vitro* experiments to be performed with a neuronally expressed gene of unknown function, and therefore observed effects are likely not representative of a full spectrum of functions that neuronal LRR membrane proteins serve. Furthermore, out of the proteins mentioned above, *in vivo physiological* role in regulating neurite growth has been established only for OMgp (Huang *et al.* 2005). Evidence suggesting *in vivo* physiological role in axonal growth and guidance is relatively strong also for NGL-1 (Lin *et al.* 2003). Several lines of evidence associates NgR1 with the regulation of neurite outgrowth in pathological settings (for review, see (Yiu and He 2006)). Other emerging functions for neuronal LRR membrane proteins are regulation of growth factor signaling (NLRR-3 and LRIG-1; **Table 2**), and synapse formation/function (SALMs).

In summary several neuronal LRR membrane proteins have important roles in regulating euronal form and function. Neuronal LRR membrane proteins can serve as ligands (*e.g.* NGL-1), as receptors (*e.g.* NgR1), or as both (*e.g.* OMgp). Most members of the neuronal

LRR membrane protein family seem to function in a non-cell-autonomous manner, but their intracellular localization and other evidence suggest that some members (Pal, LRRTM1, SALMs, Slitrks) are likely to have important cell-autonomous functions.

1.5.3 Example of neuronal LRR membrane proteins: NGL family

NGL-1 was identified as a high-affinity ligand for netrin-G1 (Lin *et al.* 2003) from an expression cloning screen. NGL-1 belongs to a three member NGL family; all family members contain nine LRRs, an IgG-like domain, transmembrane segment, and a short intracellular region, which ends carboxyl-terminally with a likely PDZ-domain binding sequence (E-T-Q-I). Surface-bound NGL-1 promotes outgrowth of embryonic thalamic axons *in vitro* whereas soluble NGL-1 inhibits outgrowth when injected into neural tube of early chick embryos. Netrin-G1 is highly expressed in mouse thalamic neurons, whereas NGL-1 is expressed in striatum and cerebral cortex – intermediate and final targets for thalamocortical axons, respectively. All this data suggests that NGL-1 is likely to play a role in axon guidance: NGL-1 is likely a surface-bound short range chemoattractant ligand that signals in netrin-G1 dependent manner to promote growth of thalamocortical axons (Lin *et al.* 2003). However, *in vivo* loss-of-function experiments to confirm this remain to be completed.

A recent study has shed light on the possible functions of NGL-2 in adulthood, suggesting that NGL-2 is a regulator of neuronal morphology and also a postsynaptic membrane protein that instructs the formation of excitatory synapses. (Kim *et al.* 2006). Specifically, NGL-2 was found to promote formation of dendritic protrusions when overexpressed, interact with PSD-95, and induce presynaptic differentiation in a non-cell-autonomous manner (when presented on the surface of HEK293 cells or on microbeads). Furthermore, NGL-2 knockdown reduced the number of excitatory synapses in cultured hippocampal neurons. The functions of NGL-2, might be at least partially mediated by netrin-G2, as the authors found that these two proteins interact physically (Kim *et al.* 2006).

Both NGL-1 and NGL-2 are prominently expressed in the adult human brain (Lin *et al.* 2003; Zhang *et al.* 2005); their possible roles in synaptic plasticity remain to be studied.

1.5.4 Example of neuronal LRR membrane proteins: SALM family

SALMs (synaptic adhesion-like molecules) compose a recently characterized five member subfamily of neuronal LRR membrane proteins. Their extracellular region contains six LRRs, an IgG-like domain, and a fibronectin type III domain. All SALMs possess a short intracellular region, and in SALMs 1-3 it ends carboxyl-terminally with a consensus PDZ-domain binding sequence. SALM1 and SALM2 were identified by yeast-two hybrid screening as proteins that bind to PDZ-domains of SAP97 and PSD-95 (Ko *et al.* 2006; Wang *et al.* 2006), respectively. SALM2 was also shown to interact with other synaptic PDZ-proteins (SAP97, Chapsyn-110, SAP102), and SALM1 was found to bind to PSD-95 as well. These interactions, as well as the plasma membrane targeting of SALM1, is dependent on the C-terminal PDZ-domain binding sequence.

Interestingly, SALMs seem to regulate multiple aspects of neuronal differentiation. Overexpression of SALM1 increases neurite outgrowth twofold (Wang *et al.* 2006) in young hippocampal (4 DIV) neurons, but not in older (14 DIV) ones (Wang *et al.* 2006). SALM1 can also interact and cluster NMDA receptors (Wang *et al.* 2006). On the other hand SALM2 was found to localize to excitatory, but not to inhibitory synaptic sites, and there it interacts with AMPA receptors to some degree, but more strongly with PSD-95 (Ko *et al.* 2006). Overexpression of SALMs seems to have various effects on spine formation, but more studies are required to resolve the partially conflicting effects observed (Ko *et al.* 2006; Wang *et al.* 2006). *In vivo* studies are necessary to confirm the reported roles of SALMs in regulating neurite morphology and function. In contrast to NGLs, SALMs do not induce the formation of presynaptic specializations in cells. Thus SALMs may function cell-autonomously whereas NGLs can function a non-cell-autonomously as well.

1.5.5 Example of neuronal LRR membrane proteins: Slitrk family

The Slitrk-family has six members, with each containing 11 LRRs, broken into two segments, in their extracellular (or in ER/Golgi lumen) region. Additionally, they have a TM segment and a short intracellular region (Aruga and Mikoshiba 2003). Their name Slitrk refers to their similarity to Slit and Trk family members, although the LRRs of Slitrks are no closer to those of Slit than to any other LRR protein, and the resemblance of Trks is limited to a presence of 0-4 tyrosine residues in the assumed intracellular region in different Slitrk family members. No evidence indicates that these tyrosines are being phosphorylated. All Slitrk family members are prominently expressed in the brain and spinal cord (Aruga and Mikoshiba 2003). Different Slitrk family members seem to have opposing effects on neurite outgrowth: In PC12 and Neuro-2a neuroblastoma cells as well as in cortical neuron cultures, overexpression of Slitrk1 promotes neurite outgrowth, whereas in same neuronal cell lines overexpressed Slitrk2 restricts neurite outgrowth (Aruga and Mikoshiba 2003; Abelson et al. 2005).

Sequence variants in Slitrk1 have been suggested to be associated with Tourette's syndrome (Abelson et al. 2005). The study by Abelson *et al.* was inspired by the finding of a chromosomal inversion in the vicinity of Slitrk1 locus. However, this inversion did not overlap with the Slitrk1 gene. Detailed sequencing of 174 individuals affected with Tourette's syndrome revealed one non-sense mutation in the ORF, and one variant in two unrelated affected individuals in the 3' UTR ('var321') that where not found in 3600 and 4296 control chromosomes, respectively. However, recent studies have failed to confirm these observations: the 'var321' was also found in non-affected Caucasian individuals (Wendland et al. 2006) and in several non-affected Ashkenazi Jews (Keen-Kim et al. 2006). Another study did not identify associated ORF variants or 'var 321' in 82 Tourette's syndrome patients (Deng et al. 2006). These results suggest that the putative Slitrk1 association with Tourette's syndrome is unlikely to be beneficial in clinical diagnostics, and highlight the importance of using precisely matched case and control populations in association analyses of rare variants.

1.5.6 Example of neuronal LRR membrane proteins: AMIGO family

AMIGO1 was discovered using differential display -method as a transcript upregulated in neurons by neuronal growth-promoting factor amphoterin (Kuja-Panula et al. 2003). AMIGO2 and AMIGO3 were successively identified by bioinformatics. All three AMIGOs share similar domain architecture: they possess six LRRs and one IgG-like domain extracellularly (or in ER/Golgi lumen), followed by a TM segment and a short intracellular region. AMIGO1 mRNA is brain-enriched whereas the two other AMIGOs are more widely expressed in the adult mouse (Kuja-Panula et al. 2003). AMIGO1 protein is particularly abundant in the fiber tracts in brain and in the neurites of cultured hippocampal neurons.

Interestingly, substrate coated with AMIGO1-Fc recombinant protein promotes hippocampal neurite outgrowth, and this effect can be reversed by soluble AMIGO1 ectodomain. Soluble AMIGO1-Fc protein also inhibits fasciculation of hippocampal neurites *in vitro*. As fasciculation involves homophilic interactions, Kuja-Panula *et al.* hypothesized that AMIGOs might show homophilic adhesion. Consistent with this hypothesis, all AMIGOs were found to co-immunoprecipitate with each other, and also to promote homophilic adhesion in bead aggregation assays. In the future studies, it would be worthwhile to quantify the binding affinities; the question of plasma membrane localization of AMIGOs should be addressed as well. In cultured neurons AMIGO2 expression is activity-dependent, and it inhibits apoptosis (Ono *et al.* 2003). Finally, AMIGO2 has also been suggested to have a role in the etiology of gastric adenocarcinoma (Rabenau *et al.* 2004). These potentially important findings warrant further studies to establish the main *in vivo* functions of AMIGO family members.

1.6 Nogo-A and Nogo-66 Receptor in axonal regeneration

This thesis addresses in part questions concerning the Nogo-66 Receptor (NgR/NgR1). Therefore, the earlier studies of this particular neuronal LRR membrane protein are discussed here in more detail. A number of studies have suggested that NgR1 mediates oligodendrocyte/myelin-to-neuron signals that inhibit CNS axon regeneration (reviewed in (McGee and Strittmatter 2003; Yiu and He 2006)).

1.6.1 Myelin inhibitors of neurite growth

Here I use the term 'myelin inhibition of neurite growth' to refer to the phenomenon that CNS myelin proteins inhibit axonal growth and regeneration, both *in vitro* and *in vivo*. CNS axons regenerate minimally after injury, but they exhibit robust growth into peripheral nerves grafted into rat CNS (David and Aguayo 1981). A similar pattern was also observed by Dr. Martin Schwab and Dr. Hans Thoenen in *in vitro* assays: the growth of rat dorsal root ganglion (DRG), sympathetic or retinal neurons is limited when grown on rat optic nerve explants (containing CNS myelin) as compared to when grown on sciatic nerve explant (containing PNS myelin) (Schwab and Thoenen 1985). This inhibitory activity was shown to be enriched in the white matter fraction of CNS (Savio and Schwab 1989). Furthermore, co-culture studies revealed that growing neurites avoid making contacts with mature oligodendrocytes (Schwab and Caroni 1988).

The observation that direct contact between the growing neurite and oligodendrocyte is needed for the growth inhibition suggested that the growth inhibition was mediated by ligand-receptor interaction (Schwab *et al.* 1993). Since the discovery of Nogo-A as the first myelin-derived inhibitor of neurite growth (below) the research on myelin inhibition of neurite growth has expanded rapidly. This expansion was facilitated by the identification of several other CNS myelin-derived molecular species that limit axonal growth. These include myelin-associated Nogo-A, MAG (myelin-associated glygoprotein), OMgp (oligodendrocyte myelin glycoprotein) and astroglial scar-derived chondroitin sulfate proteoglycans (CSPGs), and EphA4 (reviewed in (Yiu and He 2006)).

1.6.2 Identification of Nogo-A

In 1988 Caroni and Schwab identified two membrane proteins from SDS-PAGE fractionated CNS myelin that potently inhibited neurite outgrowth and 3T3 fibroblast spreading. These species were named as NI-35 and NI-250 after their apparent molecular weight (in kDa) in SDS-PAGE (Caroni and Schwab 1988). Caroni and Schwab also demonstrated that IN-1 and IN-2 monoclonal antibodies raised against these species could neutralize the inhibitory activity of CNS myelin on neurite growth and on 3T3 fibroblast spreading (Caroni and Schwab 1988). The IN-1 antibody (raised against NI-250 fraction) was also noted to cross-react with NI-35 fraction, suggesting that these two species share molecular similarities. IN-1 antibody also recognized an unidentified 50 kDa protein from CNS myelin. The molecular properties and partial amino acid sequence of NI-250 (named as bNI-220 as isolated from bovine brain and had an apparent molecular weight of 220 kDa) protein were described much later as its enrichment and purification to homogeneity required complicated methodology (Spillmann *et al.* 1998). Based on one of the amino acid sequence reads obtained, Spillmann *et al.* suggested that bNI-220 might be a member of the reticulon (RTN) family of proteins.

Soon after the study by Spillmann *et al.* three groups independently identified the gene encoding bNI-220 as Reticulon-4 (Rtn4), renamed the active splice variant as Nogo-A, and demonstrated that Nogo-A is expressed on the surface of oligodendrocytes (Chen *et al.* 2000; GrandPre *et al.* 2000; Prinjha *et al.* 2000). Rat Nogo-A is composed of 1163 amino acids, but it migrates on SDS-PAGE as a ~200 kDa protein. It contains carboxy-terminal ~200 amino acid reticulon homology domain (RHD), which is present in all Rtn family members. The RHD consists of two hydrophobic segments flanking a 66 amino acid long hydrophilic sequence. The long (~1000 amino acid) amino-terminus of Nogo-A shares no homology with other proteins. Nogo-A does not have a signal peptide and the mechanism responsible for its integration to

plasma membrane remains elusive. Based on *in vitro* neurite outgrowth assays utilizing purified fragments of Nogo-A, GrandPre *et al.* argued that neurite outgrowth activity of Nogo-A is mediated by 66 amino acid loop region (Nogo-66 segment) within the RHD, which is present in all Nogo splice variant (Nogo-A, -B, and -C), whereas Prinhja *et al.* showed that the amino-terminal region unique for Nogo-A inhibits neurite outgrowth.

1.6.3 Nogo-66 receptor (NgR1) and its ligands

Using expression cloning methodology GrandPre *et al.* identified Nogo-66 Receptor (NgR/NgR1) as a receptor mediating neurite outgrowth inhibitory activity of the Nogo-66 segment (GrandPre *et al.* 2000). The neuronal receptor for the N-terminal segment of Nogo-A is still unknown. NgR1 is a glycosylphosphatidylinositol (GPI) -anchored membrane protein that contains extracellularly eight LRRs flanked by cysteine-rich capping modules. Its crystal structure has been determined **(Figure 1)**, and as predicted, it forms a structure typical for LRR superfamily members (Barton *et al.* 2003; He *et al.* 2003). NgR1 is widely expressed in the CNS.

Soon after the identification of NgR1 as a receptor for Nogo-66, two groups independently reported that MAG (myelin associated glycoprotein), another inhibitory component of CNS myelin, binds to and signals via NgR1 (Domeniconi *et al.* 2002; Liu *et al.* 2002). The neurite-outgrowth inhibitory activity of MAG had been described much earlier (McKerracher *et al.* 1994; Mukhopadhyay *et al.* 1994). Surprisingly, a third inhibitory protein in the CNS myelin, OMgp (oligodendrocyte myelin glycoprotein), was also suggested to use NgR1 for binding and signaling (Wang *et al.* 2002). At the same time the neurite-outgrowth inhibiting activity of OMgp was described independently by another group as well (Kottis *et al.* 2002). Summary of these interactions is provided in **Figure 2**.

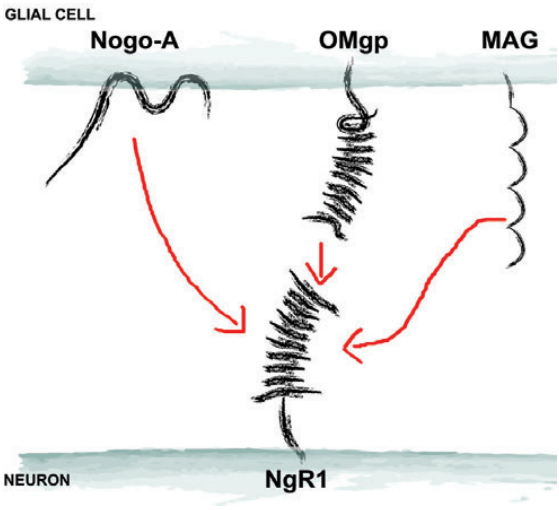

Figure 2. Proteins involved in NgR1 signaling pathways as known in June, 2002. *This diagram represents the state of knowledge before a study by Venkatesh et al. (Venkatesh et al. 2005) and a part of this thesis work* ***(III)*** was published. *Three myelin-associated ligands Nogo-A, OMgp, and MAG all bind to neuronal NgR1 with low nanomolar dissociation constants. See* ***Figure 7*** *for an up-to-date model. The schematic drawing is not in scale.*

As NgR1 is a GPI-anchored protein, it seems logical that it needs a co-receptor to conduct signals to the intracellular space. Recent years have provided evidence for several co-receptors. Initially it was noted, that p75, originally characterized as neurotrophin receptor, could mediate NgR1-signalling, and form a complex with NgR1 (Wang *et al.* 2002; Wong *et al.* 2002). However, the very limited expression of p75 in the adult CNS raised a question whether p75 is a *bona fide* co-receptor. Later, a structurally related TNFR superfamily member TROY/Taj was shown to also function as a co-receptor (Park *et al.* 2005; Shao *et al.* 2005). Additionally, a third and necessary component of the receptor complex was identified as LINGO-1, another member of neuronal LRR membrane protein family (Mi *et al.* 2004). Reported *in vivo* evidence supports the role of all of these three above mentioned co-receptors in mediating myelin inhibition of neurite growth, but their

relative importance remains unclear (for review, see (Yiu and He 2006)).

Intracellular signaling pathways engaged in NgR1 signaling are largely unclear but they seem to share similarities with signaling mechanisms used by other repulsive guidance cues. The key downstream signaling pathway might be RhoA small GTPase and its downstream target ROCK/ROCK2 (Rho-A associated kinase), which regulates actin cytoskeleton stability at the growth cone. However, also other signaling pathways (protein kinase C, EGF receptor, calcium ions are also implicated in NgR1 signaling (for review, see (Yiu and He 2006)).

1.6.4 Therapeutic potential of perturbing Nogo-A and NgR1 function

Reviewing in detail the extensive literature addressing the *in vivo* role of myelin inhibitors of neurite growth is beyond the scope of this thesis work. Briefly, Nogo-A monoclonal antibodies (Schnell and Schwab 1990; Bregman *et al.* 1995; Wiessner *et al.* 2003), NgR1 function-blocking peptide (NEP1-40) (GrandPre *et al.* 2002; Li and Strittmatter 2003), and NgR1 function-blocking recombinant protein (soluble NgR1 ectodomain) (Lee *et al.* 2004; Li *et al.* 2004; Wang *et al.* 2006) all enhanced axonal regeneration and functional recovery after spinal cord injury or stroke in rodents.

The genetic data regarding the role of Nogo-A in spinal cord regeneration, obtained by analyzing independently made mouse knock out lines, is controversial (Kim *et al.* 2003; Simonen *et al.* 2003; Zheng *et al.* 2003; Cafferty and Strittmatter 2006). Similarly, Kim *et al.* and Zheng *et al.* obtained very different results when analyzing regenerative abilities of independently prepared NgR1 knock out mouse lines (Kim *et al.* 2004; Zheng *et al.* 2005). Reconciliation of disparities is further complicated by the inherent differences in the regenerative ability of commonly used mouse strains (Dimou *et al.* 2006), and by the complex genetic background of most of the mice used in the above mentioned genetic studies.

Of these Nogo-A and NgR1 inhibitors, monoclonal antibodies against Nogo-A represent the most advanced project with the goal of treating human patients with spinal cord injury. Nogo-A antibodies have provided promising results in monkey spinal cord injury experiments (Freund *et al.* 2006). Fully humanized Nogo-A antibody is in clinical trials for acute spinal cord injury, and the phase I safety trial has been largely completed with favorable safety profile (Dr. Martin Schwab, personal communication).

1.7 Genetic basis of left-right brain asymmetry

We found that a certain LRRTM1 haplotype is associated with handedness, and also to schizophrenia in Caucasian populations. Therefore I provide here an overview on brain asymmetry, handedness, and schizophrenia.

Overall, the left and right brain hemispheres function differently during human cognition, behavior, and emotion. Handedness is a prominent example of functional hemispheric differences. Whereas dorso-ventral (D-V) and anterior-posterior (A-P) axis formation in the spinal cord and CNS has been studied intensively, few studies have illuminated the molecular basis of distinctions between the left and right hemispheres (Sun et al. 2005; Moskal et al. 2006; Sun et al. 2006).

Ninety percent of humans are more skilled at motor tasks with their right hand (i.e. left hemisphere-dominant). While approximately 95% of right-handed and 70% of left-handed people process language predominantly in the left hemisphere, spatial recognition is more dependent on the right hemisphere. At the anatomical level, differences between the hemispheres are most prominent in the frontotemporal regions, specifically along the Sylvian fissure (typically longer in the left hemisphere), and in the planum temporale (typically larger on the left side) (reviewed in (Sun and Walsh 2006)). Handedness is the most easily measured aspect of human brain asymmetry, and relative handedness can be quantified using tasks that require fine-motor skills. Studies of handedness may provide insight into the general mechanisms of brain asymmetry.

In the case of D-V and A-P axis formation in the CNS, symmetry is broken by asymmetric morphogen expression from a signaling center, which triggers graded expression of transcription factors that impose positional information on cells (Sur and Rubenstein 2005). Analogously, it is plausible that left-right axis formation results from the asymmetric expression of certain key signaling molecule(s). Studies exploring the molecular basis of left-right brain asymmetry have aimed to identify asymmetrically expressed genes by microarray or SAGE-approaches. The most detailed work by Sun *et al.* (Sun et al. 2005) identified 27 genes expressed differentially in left and right perisylvian regions of 12-week-old human brains. Sun *et al.* also confirmed by *in situ* hybridization that one of the identified genes, the transcription factor LMO4, was indeed asymmetrically expressed in developing human and mouse brain. However, as is evident from the high number of genes showing asymmetric gene expression in their study, microarray analysis seems unlikely to identify the "smoking gun" gene(s) that have an early causative role in the emergence of left-right brain asymmetry. Therefore studies on the genetics of human handedness offer a unique possibility to gain insight into this fundamental property of human brain.

1.7.1 Co-evolution of handedness and brain asymmetry

Although even drosophila (Pascual *et al.* 2004) and zebrafish (Lin and Burdine 2005) show well documented asymmetries in the anatomy and physiology of their CNS, there is no evidence to suggest that handedness, defined as a superior dexterity of either forelimb, would have emerged as a hereditary trait in other species than in humans and possibly in great apes. Generally the emergence of at least partially bipedal posture has been suggested to be necessary for enabling the use of manual gestures and handedness to emerge (Gentilucci and Corballis 2006).

Population-level handedness (better fine-motor skills) emerged very recently in evolution: the evidence for population-level handedness is limited even for apes not grown up in captivity (Sun and Walsh 2006). *Mus musculus* do not show population-level handedness, although individual mice do show consistent forelimb preference in pellet-reaching tasks (Betancur *et al.* 1991; Biddle *et al.* 1993). The strength of paw-preference lateralization is, however, genetically controlled: some inbred mouse strains show weakly whereas others (*e.g.* C57Bl/6 (Collins 1975)) show strongly lateralized paw preference. Unlike in humans, in mice the preference for left or right paw usage seems to arise as an outcome of a random process (Collins 1975).

It has been proposed that handedness and language co-emerged in evolution – that language evolved from manual gestures, gradually incorporating vocal elements (Corballis 2003). This theory suggests that hemispheric specialization for language may be predated by and may have evolved from hemispheric specializations for manual gestures used in communication. Importantly, humans show strong population-level bias for both right handedness (i.e. dominant left hemisphere) and also for the lateralization of language processing (predominantly in the left hemisphere) and the lateralization of handedness and language processing are tightly correlated: 99% of right-handed people process language in the left hemisphere. This strong correlation, and the use of hand signals to accompany speaking, has been interpreted to suggest their co-evolution. Furthermore, although great apes use vocalization for signaling, attempts to teach them to use language have been more successful when using manual signs (Gentilucci and Corballis 2006).

Genes involved in human handedness are likely to have an effect on the development of human brain left-right asymmetry. Brain asymmetry is fundamental not only to language, as detailed above, but to much of human thought and emotion (Sun and Walsh 2006). Studies on genes involved in brain asymmetry may thus yield novel insights into the biological basis of human cognition.

1.7.2 Genes and genomic regions implicated in handedness

Handedness is a partially hereditary trait (Francks et al. 2002). Studies of the genetics of human handedness were pioneered by Dr. Francks and colleagues: they performed the first genome-wide linkage screen for a quantitative measure of human handedness,

and found linkage to 2p12-q11 (Francks et al. 2002). Later the 2p12-q11 linkage was replicated in a different sample (Francks et al. 2003). They also demonstrated that this linkage was derived entirely from paternal inheritance of the 2p12-q11 region (Francks et al. 2003). This observation suggested that the underlying gene was imprinted, and inactivated or down-regulated on the maternally inherited chromosome. In a meta-analysis of 20 linkage screens for schizophrenia, 2p12-q22 was the only location to reach overall statistical significance (Lewis et al. 2003).

Previously Francks *et al.* demonstrated that the 2p12-q11 linkage to schizophrenia was also due to a paternally inherited risk allele (Francks et al. 2003). As parent-of-origin effects are unusual in the genome (currently about 50 imprinted genes are known (Morison et al. 2005)). Francks *et al.* proposed in 2003 that a single paternally expressed gene was responsible for the linkages of 2p12-q11 to handedness and schizophrenia (Francks et al. 2003).

1.8 Schizophrenia

The prevalence of schizophrenia is ~1%, and therefore it is a major health and social problem (Freedman 2003). The patient's response to current therapies is typically only partial. One of the main factors limiting the development of new drugs is the lack of useful animal models (Gogos and Gerber 2006; Powell and Miyakawa 2006). Recent advances in schizophrenia genetics have activated the field studying the etiology of schizophrenia. The current paradigm in schizophrenia research regards the defects in CNS development and plasticity as the likely etiology (Gogos and Gerber 2006). According to this paradigm, schizophrenia might be an end-result of a developmental defect, possibly of embryonic origin.

1.8.1 Schizophrenia genetics

Like handedness and brain asymmetry, schizophrenia is likely to be an etiologically complex trait with several, or many, genetic and environmental influences. Schizophrenia has an even stronger genetic component than handedness: first-degree relatives of a schizophrenic patient have ten-fold risk of developing the disease. Although each susceptibility gene allele increases the probability of schizophrenia only moderately, a combination of genetic risk factors can largely increase the likelihood of developing the disease.

Significant advances have been made in schizophrenia genetics in recent years. Currently, there is evidence associating variation in at least 13 genes to schizophrenia. Particularly certain SNPs and haplotypes in dysbindin and neuregulin-1 genes have been reported to be associated with increased risk of schizophrenia in ten and at least in nine independent studies, respectively. On the other hand, in several studies these associations were not found (for review, see (Gogos and Gerber 2006)).

1.8.2 Schizophrenia and handedness

A recent meta-analysis based on 40 studies found that schizophrenia is increased a two-fold in left- and mixed-handed population (Dragovic and Hammond 2005). Several studies have also suggested that schizophrenia is associated with reductions or reversals of normal cerebral asymmetries (see *e.g.* (DeLisi et al. 1997; Chance et al. 2005)). Thus, one can gain insight into molecular mechanisms underlying both traits by studying either of them.

1.8.3 Animal models in schizophrenia research

Over 99% of human genes have a corresponding orthologous gene in the mouse genome (Waterston *et al.* 2002). Therefore mouse genes orthologous to human schizophrenia-associated genes are likely to serve in biological roles related to their human counterparts' roles. Using mouse models to study the etiology of schizophrenia has many advantages over largely observational human studies or post mortem analysis of human brain samples. Studying animal models lacking or overexpressing genes genetically linked to schizophrenia could clarify the role of these candidate genes in schizophrenia and possibly elucidate molecular mechanisms of

schizophrenia pathogenesis. However, arguably, some schizophrenia symptoms may be human-specific (for review in animal models in schizophrenia research, see (Powell and Miyakawa 2006)).

2 AIMS OF THIS STUDY

The general aim of this study was to identify and study novel members of the neuronal leucine-rich repeat membrane protein superfamily. The specific aims were:

● To perform detailed bioinformatics analyses of identified novel members of the LRR protein superfamily (LRRTM and NGR families) [original publications **I** and **II**]

● To characterize the mRNA expression pattern of LRRTM and NGR gene family members [original publications **I**, **II**, and **IV**]

● To map the intracellular protein localization pattern of LRRTM1, a prototypic LRRTM family member [original publication **IV**]

● To characterize possible ligand-receptor interactions between identified novel NgR family members (NgR2 and NR3) and known and candidate NgR1 ligands [original publication **III**]

● To characterize in detail the interactions between NgR1 and its known ligands [original publication **III**]

● To generate knock out mouse models for the study of the functions of the selected Lrrtm gene family members *in vivo* [previously unpublished; presented here in *Results*]

● To explore the relevance of LRRTM gene families for human physiology and disease [original publication **IV**]

3 MATERIALS AND METHODS

Materials and methods used in this thesis work have been described in detail in the original articles (see **Table 3**). Here a short overview of the methods used is given; the process of generating knock out mice is described in more detail.

Experimental Procedure	Publication			
Bioinformatics	I	II	III	IV
Recombinant DNA technologies	I	II	III	IV
RT-PCR expression analysis	I	II		
In situ hybridization & neuroanatomical analysis	I	II	III	IV
Cell lines cultures			III	IV
Primary neuronal cell cultures				IV
Recombinant protein production and affinity purification			III	
Immunocytochemistry and light and confocal microscopy			III	IV
Cell-based binding assays			III	
Generation of knock out mice	See *Results*			
Human genetics and SNP genotyping				IV

Table 3. Methods used in this study.

3.1 Bioinformatics

The main bioinformatics resources used in the study are described in **Table 4**.

3.2 Recombinant DNA techniques

The expression vector constructs used in this study are described in **Table 5**.

3.3 RT-PCR expression analysis

RT-PCR analysis of mRNAs isolated from different human and mouse tissues including Lrrtm3 knock out brain was done as in **(I, II)**. In the Lrrtm3 knock out brain mRNA RT-PCR analysis, primers internal for Lrrtm3 exon 2 were used.

Bioinformatics resource	Major use	Used in publications				Reference
National Centre for Bioinformatics (NCBI), BLAST tool *(http://www.ncbi.nlm.nih.gov/blast/)*	EST identification	I	II	III		(Altschul *et al.* 1990)
Univ. California Santa Cruz (UCSC) Genome Browser *(http://www.genome.ucsc.edu/)*	Analysis of genomic structure of genes	I	II			(Kent *et al.* 2002)
Ensembl Genome Browser *(http://www.ensembl.org)*	Analysis of genomic structure of genes	I	II			(Birney *et al.* 2006)
ClustalX and NJPlot program package	Multiple sequence alignments; phylogenetics	I	II			(Chenna *et al.* 2003)
SMART (simple modular architecture research tool) tool *(http://smart.embl-heidelberg.de/)*	Protein domain identification	I	II			(Schultz *et al.* 1998)
TopPred *(http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html)*	Topology prediction of membrane proteins	I	II			(Claros and von Heijne 1994)
SignalP 2.0 *(http://www.cbs.dtu.dk/services/SignalP/)*	Signal peptide cleavage site prediction	I	II			(Bendtsen *et al.* 2004)
big-PI Predictor *(http://mendel.imp.ac.at/sat/gpi/gpi_server.html)*	GPI modification site prediction		II			(Eisenhaber *et al.* 1999)
HapMap *(http://www.hapmap.org/)*	Identification and analysis of SNPs				IV	(Thorisson *et al.* 2005)
InterPro *(http://www.ebi.ac.uk/interpro/)*	Identification of LRR superfamily members	This work				(Mulder *et al.* 2007)

Table 4. The main bioinformatics resources used in this study.

Expression construct	Elements of construct (listed from amino to carboxyl-terminus)	Described in
Myc-LRRTM1	IgK signal peptide, Myc, 6xHis, mouse LRRTM1 cDNA without signal peptide in pSECtag2a	**IV**
Myc-Lingo-1	IgK signal peptide, Myc, 6xHis, mouse Lingo1 cDNA without signal peptide in pSECtag2a	**IV**
Myc-MAG	IgK signal peptide, Myc, 6xHis, mouse MAG cDNA without signal peptide in pSECtag2a	**III**
MYC-TLR4	IgK signal peptide, Myc, 6xHis, mouse MAG cDNA without signal peptide in pSECtag2a	**III**
Myc−NgR	Mouse NgR1 (aa 27-473) in pSecTag2-Hygro	(Fournier *et al.* 2001)
Myc-Ngr2/NGRL3	IgK signal peptide, Myc, 6xHis, mouse NgR2 cDNA without signal peptide in pSECtag2a	**III**
Myc-Ngr3/NGRL2	IgK signal peptide, Myc, 6xHis, mouse NgR3 cDNA without signal peptide in pSECtag2a	**III**
FLAG-NgR mutants	Library of 3xFLAG tagged human NgR mutants in p3xFLAG-CMV™-14 (Sigma)	**III**
AP-Nogo-66	Rat Nogo66 in pAPtag5	**III**
AP-Y4C	human Nogo-A, aa 950-1018, in pcAP6	(Hu *et al.* 2005), **III**
AP-Nogo24	human Nogo-A, aa 995-1018, in pcAP6	(Hu *et al.* 2005), **III**
AP-Nogo-C39	Human Nogo-A c-term. 39 aa in pAPtag5	**III**
AP-MAG	AP-MAG ectodomain fusion (mouse) in pcAP6	(Liu *et al.* 2002), **III**
MAG-AP	MAG ectodomain (mouse) fusion in pAPtag5	**III**
AP-OMGP	AP-OMGP ectodomain fusion (mouse) in pAP-5	(Wang *et al.* 2002), **III**
AP-Lingo-1	AP-Lingo ectodomain fusion (human)	(Mi *et al.* 2004), **III**
AP-RTN1-66	66 amino acid RHD loop region of mouse RTN1 in pAPtag5	**III**
AP-RTN2-66	66 amino acid RHD loop region of mouse RTN2 in pAPtag5	**III**
AP-RTN3-66	66 amino acid RHD loop region of mouse RTN3 in pAPtag5	**III**

Table 5. Expression vector constructs used in this study.

3.4 *In situ* hybridization

Fresh-frozen mouse sections were analyzed by *in situ* hybridization using [α-35S]UTP-labeled cRNA probes. The probes used are described in **Table 6**.

Probe	Used in publication			
Mouse LRRTM1, ORF nt 1–448	**I**			**IV**
Mouse LRRTM1, ORF nt 1131–1566	**I**			**IV**
Mouse LRRTM2, ORF nt 1–471	**I**			
Mouse LRRTM2, ORF nt 1045–1545	**I**			
Mouse LRRTM3, ORF nt 1–541	**I**			
Mouse LRRTM3, ORF nt 1061–1542	**I**			
Mouse LRRTM4, ORF nt 1–431	**I**			
Mouse LRRTM4, ORF nt 1119–1554	**I**			
Mouse NgR1, whole ORF		**II**		
Mouse NgR2/NgRL3, ORF nt 480–1263		**II**		
Mouse NgR3NgRL2, ORF nt 1-491		**II**		
Mouse NgR3NgRL2, ORF nt 804-1338		**II**		
Mouse RTN1, overlaps with RHD		**II**	**III**	
Mouse RTN2, overlaps with RHD		**II**	**III**	
Mouse RTN3, overlaps with RHD		**II**	**III**	
Mouse RTN4, overlaps with RHD		**II**	**III**	

Table 6. In situ hybridization probes used in this study. *RTN1-4 probes contain ORF nucleotides 1778-2341, 808-1413, 156-711 and 19-597 of GenBank clones NM_153457, NM_013648, NM_053076, and NM_024226 for RTN1–4, respectively. The probes were designed to recognize all splice variants possessing RHD. All indicated segments were cloned into pCRII-TOPO vector (Invitrogen).*

3.5 Cell culture

The cell lines (HEK293T, Cos7, Neuro-2a) were obtained from ATCC (American Type Culture Collection) and grown in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco) supplemented with 10% FCS (fetal calf serum) and antibiotics.

3.6 Neuronal cell culture

Cerebellar granular neurons (CGNs): CGNs were isolated from 6-9 days old rats by mechanical fragmentation of the isolated cerebellum, followed by trypsin and DNaseI treatment and Percoll gradient centrifugation. CGNs were cultured in Neurobasal-A (Invitrogen) supplemented with B27 (Invitrogen), 5% FCS and 2mM L-glutamine.

Dorsal root ganglia (DRG) neurons: isolated dorsal root ganglia from 3-5 weeks old rats were digested with collagenase and mechanically triturated. DRG neurons were cultured in Neurobasal-A supplemented with 10% FCS, 2 mM L-glutamine and 100 nM nerve growth factor (NGF).

Cortical neuron cultures: mouse E17 cerebral cortex neurons were isolated by enzymatic

(Papin, DispaseII) digestion and mechanical trituration of the cerebral cortex.

All neurons were grown on poly-L-lysine and laminin coated slides.

Electroporations were performed with Amaxa Nucleofector System following the manufacturer's (Amaxa GmbH) instructions.

3.7 Recombinant protein production and affinity purification

HEK293T cells were transfected with appropriate plasmids encoding secreted AP-fusion proteins. The conditioned media was collected 3-7 days later, sterile-filtered and buffered with 20 mM HEPES, pH 7.4. Single-use aliquots were stored at -20˚C or -70˚C. Protein purification was performed with nickel-nitrilotriacetic acid (Ni-NTA) affinity resin according to manufacturer's guidelines (Qiagen). The integrity and amount of AP-proteins were estimated by PAGE followed by Western blotting or Coomassie Blue staining, and by pNPP (p-nitrophenyl phosphate) alkaline phosphatase (AP) activity assays.

3.8 Immunocytochemistry and light and confocal microscopy

Immunocytochemical staining were performed using commercially available antibodies as described in **(III, IV)**.

3.9 Cell-based ligand binding assays

Cos-7 cells were used in the binding assays because they have large surface area and they are highly adherent. Routinely 96-well plate format was used in binding assays. Cells were transfected with Fugene6 (Roche). 48 hrs after transfection cells were washed with Hank's Balanced Salt Solution (HBSS) and subsequently incubated with AP-protein conditioned media or purified ligands in HBSS + 0.05% BSA for 3 hrs. The cells were then washed extensively with cold HBSS, fixed, and then incubated at 67˚C for 14 hrs to inactivate endogenous alkaline phosphatase. Finally, the bound AP-fusion protein was visualized with BCIP/NBP (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) it 100 mM Tris, pH 9.5. Binding was quantified by acquiring multiple images per condition, and analyzing the signal intensity with ImageJ software (NIH).

3.10 Generation of knock out mice

Mouse Lrrtm1 and Lrrtm3 genes were chosen for gene targeting. A single exon encodes the whole open reading frame (ORF), or the whole ORF minus nucleotides encoding the first amino acid in Lrrtm1 and Lrrtm3, respectively. This ORF-coding exon was chosen for targeting in both cases.

3.10.1 Targeting constructs

Plasmids backbones used in the construction of replacing cassettes were obtained from Dr. Peter Mombaerts (Rodriguez *et al.* 1999). They contain the following elements in 5' -> 3' order: IRES-tauLacZ-loxP-neo-LoxP (used for Lrrtm1) and IRES-tauGFP-loxP-neo-LoxP (used for Lrrtm3). The targeting constructs contained flanking arms varying in length from 1.2 kb to ~4 kb.

3.10.2 Embryonic stem cell targeting

Embryonic stem cell (ES cell) targeting was performed by electroporating linearized constructs into R1 ES cells. After G-418 selection about 200 colonies were picked per targeting event, and analyzed by Southern blotting for the presence of homologous recombination.

3.10.3 Mouse breeding schemes

Good percentage chimaeras were used for germline transmission. Obtained heterozygous mice were mated to generate knock out mice and RT-PCR was used to verify the correct deletion of mRNA. Lrrtm3 null allele has been transferred to C57Bl/6 background (> 9 times of backcrossing of heterozygous mice); Lrrtm1 null allele is similarly being backcrossed to C57Bl/6 genetic background.

3.10.4 Removal of the neo-cassette

Before backcrossing, heterozygous mice were mated with transgenic mice expressing Cre-recombinase in testes. The correct removal of neo-cassette was confirmed by PCR.

3.11 SNP genotyping and human genetics

To study genetic variation in LRRTM1 locus in humans TaqMan® SNP genotyping assays (Applied Biosystems) were used to genotype SNPs rs1446109 and rs723524 from 1151 Han Chinese individuals, belonging to 282 pedigrees. Real-time PCR reactions were performed using ABI 7900 instrument (Applied Biosystems); the measured fluorescence intensities of the PCR products were used to discriminate the alleles. The statistical analysis was mainly performed by Dr. Clyde Francks

4 RESULTS AND DISCUSSION

4.1 Characterization of LRRTM and NGRL gene families

Based on their similarity to LRRs of TrkA, several EST clones encoding a gene that was later named as LRRTM1 (leucine-rich repeat transmembrane neuronal) were identified **(I)**. cDNA clones encoding other members of the LRRTM family were identified by bioinformatics based on their similarity to LRRTM1. I found four members of human LRRTM gene family, as well as their mouse orthologues, Lrrtm1-4. When referring to human and mouse genes, capital letters are used.

I also discovered two novel members of the NGR family in humans and in mouse. These NGR family members (NGR2/NGRL3 and NGR3/NGRL2) were initially discovered by bioinformatics based on their similarity to LRRTMs **(II)**. Since NGR2/NGRL3 and NGR3/NGRL2 are significantly more closely related to each other than to NGR1, and since they do not bind Nogo-66, I classify them here as members of their own subfamily: 'NGR like (NGRL)-family'. When referring to all three genes, I use the name 'NGR-family'. When referring to NGR genes I use capital letters, and when referring to proteins, I use established format: NgR1, NgR2/(NgRL3) and NgR3/(NgRL2).

4.1.1 The emergence of LRRTM and NGR gene families

Orthologous LRRTM and NGR family genes can be found in all vertebrates with available comprehensive sequence data. No genes orthologous to LRRTMs or NGRs are present in invertebrate (*e.g. Drosophila melanogaster, C. elegans*) or in *Ciona intestinalis* - hemichordate genomic sequence databases.

All LRRs in LRRTMs and in NGR1 and NGR3 are encoded by a single exon. In NGR2 (NGRL3) the LRR-encoding region is split into two exons.

The commonly intron-free structure of LRR-encoding segments of genes is also consistent with the observation that all LRRs and the flanking cysteine-rich capping modules commonly create a single functional protein domain (see *e.g.* **(III)**) therefore likely serving as a single modular unit in protein evolution. The paucity of introns could also suggest that LRRTM and NGR genes emerged by retrotransposition. Synteny analysis, however, reveals that at least LRRTM1-3 and NGRLs emerged by segmental chromosomal duplications (see below).

The paucity of introns limits the opportunities for alternative splicing. As functional diversity can be generated by gene duplication or by alternative splicing, one could assume these processes to be functionally interlinked. Strikingly, by analyzing human and mouse genomes, Kopelman *et al.* found an inverse correlation between the size of a gene's family and its use of alternatively spliced isoforms (Kopelman *et al.* 2005). Thus, these seemingly disparate observations could offer a set of complementary explanations for the current LRRTM and NGR gene and gene family structure.

LRRTM1, LRRTM2, and LRRTM3 genes in human and mouse are located within the largest intron of different α-catenin gene family members, and encoded by opposite strand of the DNA than the associated α-catenin genes. LRRTM4 seems to have arisen as the latest member of LRRTM family by smaller segmental duplication of ancestral LRRTM3 locus, as it is clearly most closely related to LRRTM3 and is not located within an α-catenin gene.

This nested localization of LRRTM and α-catenin genes clearly suggests and intriguing scenario of co-evolution by segmental duplication, and it also suggests that possibly transcriptional regulation between these gene pairs is interdependent. Nested gene pairs are relatively common in the human genome: 373 such gene pairs have been reliably annotated; two-thirds of them are encoded by opposite strands of DNA (Yu *et al.* 2005). Interestingly, inverse correlation in the mRNA expression levels has been reported for the members of some nested gene-pairs (Yu *et al.* 2005; Seidl *et al.* 2006). This observation could be explained by transcriptional interference

between the partners (Yu *et al.* 2005). Research of this potentially important mechanism should receive more attention in the future, as the current mechanistic studies have been limited to Igf2r-Air non-coding RNA (ncRNA) gene cluster (Seidl *et al.* 2006).

The evolutionary origin of the NGR family is more challenging to trace: Paralogous POV and serpin family genes flank NGR2 and NGR3 and NGR2 and NGR3 share functional (ligand-binding properties) and amino acid sequence similarities closer to each other compared to NGR1. All these suggest that segmental duplication of ancestral NGR2/3 locus was the last duplication event in this gene family.

4.2 Analysis of the primary, secondary and tertiary structure of the proteins encoded by LRRTM and NGR gene families

Features of LRRTM and NGR proteins can be analyzed by bioinformatics. Each LRRTM gene encodes a protein with a signal peptide, ten LRRs, transmembrane segment, and a short region that, by bioinformatics, is localized in the cytoplasmic space. Protein structures of NgRs are highly related to those of LRRTMs: NgRs possess a signal peptide, eight LRRs, and a GPI-modification site that eventually anchors the mature proteins to the plasma membrane **(Figure 3)**.

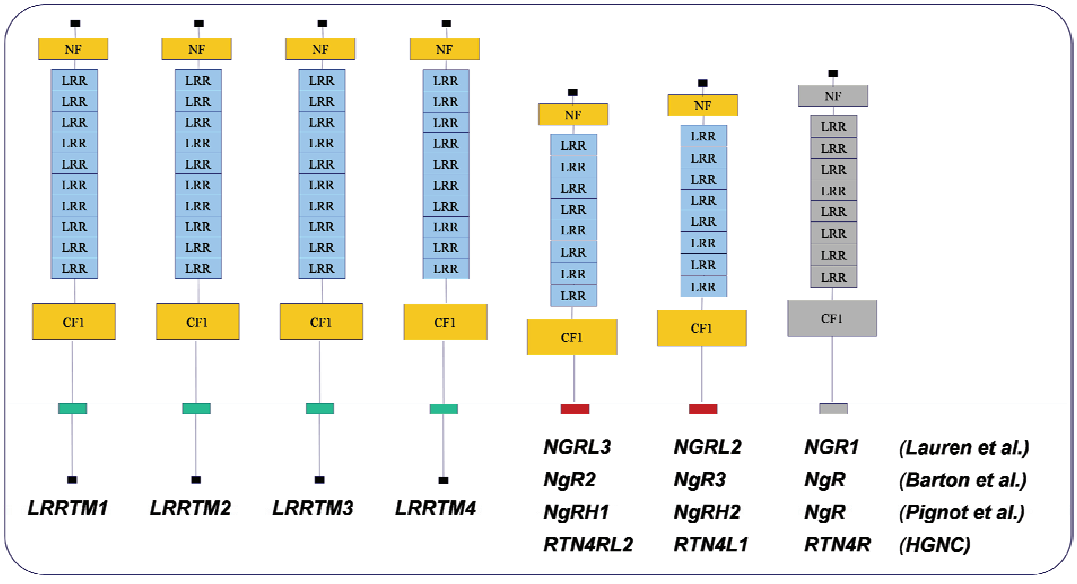

Figure 3. Schematic structure of LRRTM and NgR family proteins. *The different nomenclature suggested for NGR family members is shown. In this work the nomenclature suggested by (Barton et al. 2003) is used, except when specifically referring to the NgRL-subfamily. The alternative nomenclatures for NGR family were suggested around the same time by (Pignot et al. 2003) and in **(II)**. NF and CF1, cysteine-containing amino- and carboxyl-terminal flanking domains, respectively. Extracellular (or ER/Golgi lumen) space is above and cytoplasmic space is below the depicted lipid bilayer. RTN4R, reticulon-4 receptor; HGNC, Human Genome Organization Gene Nomenclature Committee.*

```
human LRRTM1    451 KCFPASLRQLRQCFVTQRRKQKQTMHQMAAMSAQEYYVDYKPNHIEGALVIINEYGSCTCHQQPARECEV  522
mouse LRRTM1    451 KCFPASLRQLRQCFVTQRRKQKQTMHQMAAMSAQEYYVDYKPNHIEGALVIINEYGSCTCHQQPARECEV  522
human LRRTM2    446 KCCPPTLRRIRQCSMVQNHRQLRSQTRLHMSNMSDQGPYNEYEPTH-EGPFIIINGYGQCKCQQLPYKECEV  516
mouse LRRTM2    445 KCCPPTLRRIRQCSMIQNHRQLRSQTRLHMSNMSDQGPYNEYEPTH-EGPFIIINGYGQCKCQQLPYKECEV  515
human LRRTM3    443 KRYPASMKQLQQRSLMRRHRKKKRQSLKQMTPS-TQEFYVDYKPTNTETSEMLLNGTGPCTYNKSGSRECEV  513
mouse LRRTM3    444 KRYPASMKQLQQRSLMRRHRKKKRQSLKQMTPG-TQEFYVDYKPTNTETSEMLLNGTGPCTYSKSGSRECEV  514
human LRRTM4    448 KRYPASMKQLQQHSLMKRRRKKARESERQMNSP-LQEYYVDYKPTNSETMDISVNGSGPCTYTISGSRECEV  518
mouse LRRTM4    448 KRYPASMKQLQQHSLMKRRRKKARESERQMNSP-LQEYYVDYKPTNSETMDISVNGSGPCTYTISGSRECEV  518
                      *.:::::::*  : :.:::   ::  :*     *  * :*:*.: *   : :*  * *.      :****
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Figure 4. Predicted functional elements in the cytoplasmic segment of LRRTMs. *Shown is the amino acid sequence alignment of intracellular parts of human and mouse LRRTMs. For LRRTM3 and LRRTM4 the short isoforms are shown. Identical amino acids are indicated with asterisks; physicochemically highly similar ones are indicated with colons; somewhat similar ones with dots. Putative dibasic ER-retention signals are highlighted on black background, as are the amino acids contributing to the putative ER-export motifs (di-acidic (commonly D/E-x-D/E) or di-hydrophobic (commonly FF/FY/LL/VV)). Identical ER-retention and ER-export signals are described earlier in the literature. Note that other LRRTMs also have sequences that are closely related to the marked ER-export and ER-import motifs. Putative protein kinase C (PKC) phosphorylation sites are marked on grey background, and putative protein kinase A (PKA) phosphorylation sites are underlined. Carboxyl-terminal motif resembling PDZ-protein binding motif is underlined as well.*

```
h-LRRTM1     522 V                                                                    52
m-LRRTM1     522 V                                                                    522
h-LRRTM2     516 V                                                                    516
m-LRRTM2     515 V                                                                    515
h-LRRTM3     513 V                                                                    513
h-LRRTM3-1   513 IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMETHLETELDLSTITTAGRISDHKQQLA  581
m-LRRTM3     514 V                                                                    514
m-LRRTM3-1   514 IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMESHLETELDLSTITSAGRISDHKPQLA  582
h-LRRTM4     518 V                                                                    518
h-LRRTM4-1   518 MPHHMKPLPYYSYDQPVIGYCQAHQPLHVTKGYETVSPEQDESPGLELGRDHSFIATIARSAAPAIYLERIAN  590
m-LRRTM4     518 V                                                                    518
m-LRRTM4-1   518 IPHHVKPLPYYSYDQPVIGYCQAHQPLHINKAYEAVSIEQDDSPSLELGRDHSFIATIARSAAPAIYLERITN  590
                 :*  ::  .: :****.*.** .*: *   *.:*: : *:  .. **   * * *:: .* :     *
```

Figure 5. Alternative carboxyl terminus of LRRTM3 and LRRTM4 proteins ("long isoforms"). *Amino acid similarity is depicted as in* ***Fig. 4****. m, mouse; h, human.*

```
Homo sapiens        IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMETHLETELDLSTITTAGRISDHKQQLA*
Macaca fascicularis IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMETHLETELDLSTITTAGRISDHKQQLA*
Mus musculus        IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMESHLETELDLSTITSAGRISDHKPQLA*
Rattus norvegicus   IPLSMNVSTFLAYDQPTISYCGVHHELLSHKTFETNAQEDTMESHLETELDLSTITSAGRIGDHKPQLA*
Canis familiaris    IPLSMNVSTFLAYDQPTISYCGVHHELLSHKSFETNAQEDTMETHLETEVDLSTITTAGQISDHKQQLA*
Gallus gallus       IPLAMNVSTFLAYDQPTISYCGVHHELLAHKAYNSNTQEEAVETHLETDLDLSTITTAARIKEHSQQLA*
```

Figure 6. Alternative carboxyl terminal splice variant ("long isoform") of LRRTM3 is conserved in evolution. *Sequence comparison between several vertebrates is shown. TBLASTN-searches were used to identify homologous genomic sequences.*

4.2.1 Analysis of the cytoplasmic segment of LRRTMs

Several motifs could be identified in the cytoplasmic segment of LRRTMs by bioinformatics (**Figure 4**). Importantly, both ER-import and ER-export motifs could be identified, suggesting a complex regulation of intracellular trafficking. Different targeting signals could be masked or exposed at different times, as shown *e.g.* for NMDA receptor (Scott *et al.* 2001).

I also present here an analysis of previously uncharacterized alternative splice variants of LRRTM3 and LRRTM4 that encode proteins with an alternative carboxyl terminus (**Figure 5**). The presence of these long isoform splice variants is supported by several independent EST clones and full-length cDNA clones (*e.g.* GenBank entries BC111492 for human and BC113178 for mouse LRRTM3, and BC0373216 for mouse LRRTM4). These alternative transcripts have been conserved in evolutionary time-scale, as shown for LRRTM3 in **Figure 6**. This further suggests the importance of the intracellular region of LRRTMs for their proper function and/or localization.

4.3 Localization of LRRTM and NGR family mRNAs and proteins

Tissue distribution of LRRTM and NGR family mRNAs was examined by RT-PCR and *in situ* hybridization. Transgene reporters were also utilized to study the expression of Lrrtm3 and Lrrtm1. Cellular level protein localization was studied by expressing LRRTM1 and various other expression constructs in non-neuronal cells and in neurons.

4.3.1 mRNA expression pattern of mouse and human LRRTMs and NGRs

In mouse brain, Lrrtm and NGR family members show somewhat similar mRNA expression profiles; importantly, all members of these gene families are robustly and predominantly expressed in the adult mouse brain. The mRNA expression of all members of these two gene families is also developmentally upregulated in the CNS, and it reaches steady-state levels within the first days after birth. However, each Lrrtm and NGR family member has unique, though partially overlapping mRNA expression pattern (*e.g.* in the hippocampus, cerebellum, and olfactory bulb the expression patterns differ dramatically). This implies that each member of these gene families might have partially non-redundant function(s) in the brain. The dramatic differences in expression patterns suggest that individual Lrrtm and NGR gene's regulatory regions have been under significant selective pressure. Overall, the expression of Lrrtms and NGRs suggests that they function not only in the developing but also in the adult CNS.

On the other hand, in organs other than brain, the mRNA expression profiles of these genes differ dramatically from each other: whereas mRNA expression of NGR family members is largely restricted to brain, Lrrtm1, Lrrtm4, and especially Lrrtm2 are also significantly expressed in other organs. This suggests that LRRTMs serve a cell biological role not unique to neurons and/or that they have multiple functions in different cell types.

Since we found that the LRRTM1 locus is genetically linked to handedness and schizophrenia, we analyzed in further detail the expression of Lrrtm1 mRNA during development. *In situ* hybridization analysis of E12.5 and E15.5 mouse embryos revealed specific but widely distributed Lrrtm1 expression in the developing CNS, including spinal cord, mesencephalon, pons, and developing forebrain. Although it remains unclear which brain regions are critical for the aforementioned human phenotypes, both handedness and schizophrenia originate during development. We found that at the above indicated developmental time points Lrrtm1 mRNA is expressed predominantly in post-mitotic neurons of the developing nervous system. Recently, Haines and Rigby reported that Lrrtm1 is also expressed in the neural progenitors of the E9 rostral neural tube (Haines and Rigby 2007).

4.3.2 LRRTMs and NGRs are expressed neuronally

High magnification analysis of *in situ* hybridization samples revealed that Lrrtms and NGR family members are expressed selectively in neurons.

Interestingly, in nearly all reported studies the CNS-expressed LRR-containing membrane proteins were found to be expressed in neurons. The known exceptions are OMgp, which is expressed in oligodendroglia-like cells (Huang et al. 2005), but also prominently in neurons (Habib *et al.* 1998). In addition, Lib/LRRC15 is expressed at least in astrocytes (Satoh *et al.* 2005).

4.3.3 Subcellular localization of LRRTM1 and NgR family members

All NgR family members are transported to the plasma membrane, at least when overexpressed in non-neuronal cells (Barton *et al.* 2003). In contrast, I found that LRRTM1 is not transported to plasma membrane, but it co-localizes with ER-markers. When expression vector construct encoding myc-LRRTM1 is transfected into E17 mouse cortical neurons or P18 rat DRG neurons, or into neuronal (neuro-2a), or non-neuronal (Cos-7) cell lines, no myc-immunoreactivity was detected on the plasma membrane. In contrast, in control experiments strong cell-surface related neuronal LRR membrane protein myc-LINGO1 was observed **(IV)**.

In fixed and permeabilized Cos-7 cells myc-LRRTM1 co-localized with ER-marker (dsRED2-ER). In neurons (P18 DRGs), LRRTM1 is localized to both the cell soma and to the neurites; in neurites it is also localized to lamellipodia of the growth cones **(IV)**.

Further supporting the intracellular localization of LRRTM1, when transfected into HEK293T cells, none of the six tested alkaline phosphatase (AP) fusion constructs containing LRR-domain of LRRTM1 in various constellations with AP, is secreted into conditioned media in detectable amounts, but they accumulate intracellularly. This result differs dramatically from the behavior of NgR1-AP, NgR2-AP, NgR3-AP, and LINGO1-AP constructs (encoding LRR domain-AP fusion proteins) that, when transfected into cells, produce proteins that are avidly secreted into conditioned media (unpublished observation).

The subcellular localization of LRRTM1 suggests that LRRTM1 could control protein trafficking and thereby neuronal differentiation. In most reported studies, neuronal LRR membrane proteins have been found to be transported to the plasma membrane. In addition to LRRTM1, Pal is also localized intracellularly (Gomi et al. 2000). It should be noted that endogenous subcellular localization of most neuronal LRR membrane proteins is at best poorly characterized and nearly all data regarding intracellular localization is obtained by overexpressing the protein of interest.

4.4 Mapping the NgR family interactome

As a part of this thesis work, interactions between NgR family members and known NgR1 ligands were analyzed in detail. This study was prompted by a need to better understand the functions of NgR2 and NgR3, and to explore the possible redundancy in the signaling of myelin inhibitors of neurite growth. The molecular interactions were determined by using cell-based binding assays in combination with alkaline phosphatase conjugated ligands.

4.4.1 Interactions between multiple reticulons and NgR1

Reticulon-4/Nogo-A belongs to a four member reticulon family; the homology between family members is limited to the 200 amino acid reticulon (RTN) homology domain (RHD), which consists of a 66 amino acid hydrophilic sequence stretch flanked by two hydrophobic segments (Oertle *et al.* 2003). In addition to their neuronal expression, the high amino acid sequence similarity of the RHD between RTNs, and the high amino acid sequence similarity between different NgR family members, led us to propose earlier that different RTNs could interact with different NgR family members **(II)**. I tested this hypothesis using AP-tagged forms of different RTN-66 regions, and found that RTN2-66 and

RTN3-66 interact with NgR1 with a similar low nM affinity than Nogo-66 **(III)**.

NGR1, *Rtn*2 and *Rtn3* mRNA expression patterns in the brain are largely overlapping, and NGR1 is expressed in a sub-set of *Rtn2* and *Rtn3* positive neurons **(II)**. I also found that all Rtns are most prominently expressed in neurons. Thus the detected RTN2-66:NgR1 and RTN3-66:NgR1 interactions could participate in neuron-to-neuron signaling or regulate neuronal formation and function in a cell-autonomous fashion.

The detected RTN2-66:NgR1 and RTN3-66:NgR1 interactions could also have a role in NgR1-mediated myelin inhibition of axonal growth. With this regard, I studied in detail the expression of all *Rtn* mRNA splice variants encoding proteins with RHD domains in the adult mouse spinal cord. I found that *Rtn3* mRNA is expressed in the same glial cell population of spinal cord white matter as Nogo-A mRNA **(III)**. Thus it is possible that this detected molecular interaction has a role in myelin inhibition of neurite growth in the mouse spinal cord.

Previously it has been noted that GST-RTN1-66 and GST-RNT3-66 preparations do not cause collapse of developing chick sensory neuron's growth cone in an *in vitro* assay. However, the functions of other RTN-66 domains should be tested using other and more robust experimental paradigms of neuronal growth. The GST-fusion recombinant proteins used in the earlier studies could also be misfolded as ~95% were found to be insoluble. Nevertheless, these results thus suggest that RTN2-66 and RTN3-66 segments could antagonize Nogo-66 -induced NgR1 signaling.

4.4.2 MAG is a high affinity ligand for NgR2

Similarly as above, I systematically studied the interactions between MAG and NgR family members. I found that MAG interacts not only with NgR1 but also with NgR2 **(III)**. My results here are largely in agreement with earlier studies by Venkatesh *et al.* (Venkatesh *et al.* 2005), who has also shown that MAG:NgR2 interaction likely has a role in regulating neurite outgrowth *in vitro*.

4.4.3 Characterization of OMgp-MAG interaction

OMgp has also been reported to function as a high-affinity ligand for NgR1 (Wang et al. 2002). I aimed to confirm and extend these observations by studying the possible interactions between OMgp and NgR family members. While I did not detect interaction between OMgp and NgR2 or NgR3, I did find that OMgp interacts with MAG **(III)**. OMgp's affinity to MAG is higher than its affinity to NgR1 **(III)**.

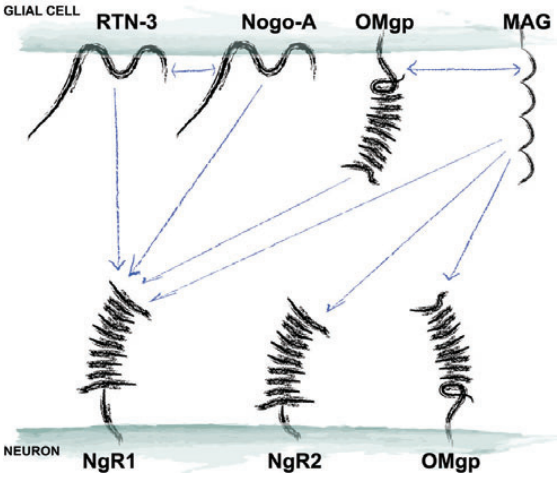

Figure 7. Summary of observed molecular interactions involving NgR family members. *This schematic figure illustrates the proposed glia-to-neuron signaling mechanisms. Since no Rtn2 expression has been detected in glial cells, it is omitted from the figure, although it binds to NgR1. Not drawn in scale. Reprinted from **(III)**.*

The reportedly low levels of OMgp expression in glial cells, and its prominent expression in neurons (Habib *et al.* 1998) suggests that a ternary complex consisting of OMgp, MAG and NgR1 could regulate specific aspects of oligodendrocyte-neuron-interactions. Neuronal OMgp could regulate glial cell functions by interacting and signaling via glial-cell expressed MAG as well. The summary of molecular interactions observed is provided in **Figure 7**.

4.5 Mechanistic insight into how NgR1 interacts with multiple ligands

How can NgR1 interact with such a high affinity with multiple structurally divergent ligands? To answer this question, I aimed first to map the nature of Nogo-A:NgR1 interactions in more detail. Then we aimed to systematically map the binding sites of multiple myelin ligands in NgR1 using a NgR1 alanine-mutant library consisting of NgR1 expression constructs with one or multiple solvent-accessible surface residues mutated to alanine. The AP-fusion ligand cell-based binding assay is versatile and amenable for scaling up making it feasible to test multiple ligands with the extensive NgR1 mutant library. The construction as well as most of the analysis of NgR1 alanine-mutant library was done by Dr. Feghua Hu, and Joanna Chin and Ji Liao.

4.5.1 Multiple sites in Nogo-A interact with NgR1

Nogo-A does not have a signal sequence, and the mechanism how it is inserted into the oligodendrocyte lipid bilayer in unknown. The putative transmembrane/intramembrane domains in Nogo-A are of unusual length (35 and 36 amino acids), suggesting that they may span the lipid bilayer twice. A recent report proposed that all three segments of Nogo-A are presented *in cis* on the same side of lipid bilayer (Voeltz *et al.* 2006). Most Nogo-A is present in the ER. Interestingly, as Nogo-A contains a carboxyl-terminal ER-retention signal, we reasoned that Nogo-A could escape ER retention by presenting this segment to the ER lumen/extracellular space. Thus, we sepculated that carboxyl-terminal amino acids of Nogo-A could also contribute to NgR1 binding. I tested this hypothesis by preparing AP-fusion protein containing carboxyl-terminal 39 amino acids of Nogo-A, and found that this segment of Nogo-A interacts with NgR1 with low nM affinity **(III)**. For the schematic structure of Nogo-A indicating the nomenclature used, see **Figure 8**.

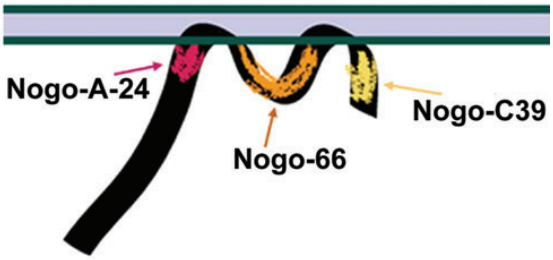

Figure 8. Schematic structure of Nogo-A indicating the nomenclature used. *The amino-terminus is left and the extracellular space is down. The three NgR1 binding segments are highlighted. Nogo-66 fragment binds to NgR1 with the highest affinity, whereas Nogo-A-24 and Nogo-C39 bind with somewhat lower affinity. The presented topology on the plasma membrane is consistent with the observations; the topology of Nogo-A on the plasma membrane has not been experimentally determined. It is possible that several conformations exist. Not drawn in scale; in vivo the amino terminal segment is ~x30 longer than carboxyl terminal segment. Reprinted from **(III)**.*

4.5.2 Myelin ligands' binding to NgR1 requires overlapping but partially separate residues

By using a library of 74 NgR1 mutants and AP-fusion ligands (Nogo-66, Nogo-Y4C, Nogo-C39, MAG, OMgp) the residues in NgR1 critical for ligand binding were determined **(III)**.

A large number of alanine-substituted NgR1 mutants bound all known ligands with similar affinity as wild-type NgR1. These residues were mostly located on the convex side of the NgR1. This suggests that the convex side is not a primary interface for known ligand-NgR1-interactions. This is also the case in other determined LRR receptor-ligand -interactions (internalin:E-cadherin (Schubert *et al.* 2002); glycoprotein Ibα:von Willebrand factor (Huizinga *et al.* 2002)).

A second set of NgR1 mutants lost the ability to bind all ligands completely. Interestingly, all of these residues are clustered on the middle of the concave side of NgR1's LRR domain. This suggests that all ligands utilize this central binding region. It is possible that subtle differences in critical binding regions between different ligands do exist, but the resolution of the screen did not allow us to identify them.

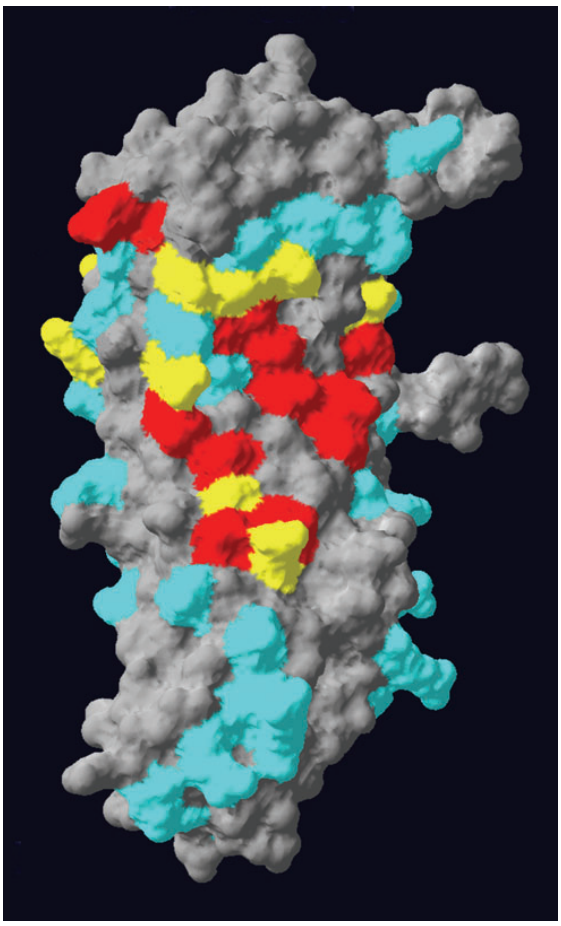

Figure 9. Residues in concave side of NgR1's LRR domain are critical for Nogo, MAG and OMgp binding. *The model is based on the analysis of 74 alanine substituted NgR1 mutants. Residues required for the binding of all three ligands (red), some ligands but not others (yellow), and not required for ligand binding (blue) are highlighted. Reprinted from* ***(III)****.*

A third set of NgR1 mutants selectively lost the ability to bind some but not all ligands, either completely or partially. These residues were scattered largely around the central binding region defined by the second set of NgR1 mutants (above). The model for ligand:NgR1 interactions based on these results is presented in **Figure 9**.

The identification of a central binding region in NgR1 shared by multiple ligands may facilitate the design and development of small molecule therapeutics blocking the binding of all NgR1 ligands. This kind of general NgR1 antagonist might promote axonal regeneration after CNS injury. Projects to screen chemical libraries with the aim of identifying small molecules capable of blocking NgR1's interactions with its ligands are ongoing at Dr. Strittmatter's laboratory.

It should be noted that as in nearly all molecular biology experimentation, the summary of binding data presented here is simply a model consistent with the current existing observations. The current gold standard in defining residues critical for protein-protein -interactions is to combine X-ray crystallographic methods with confirmatory mutagenesis analysis. No crystal structures for NgR1 in complex with any of its ligands have been reported. However, several observations suggest that the presented model is relatively accurate. First, the alanine-mutated NgR1-constructs included in the analysis were expressed in a similar fashion (similar expression level and plasma membrane localization) as wild-type NgR1. Second, a large collection of mutants did not disrupt the binding of any ligands further suggesting that mutagenesis in general is not detrimental to NgR1's folding. Third, many NgR1 mutants lost ability to bind selectively to some, but not to all of the ligands. Thus the remaining binders provide an internal control that the overall folding of mutant NgR1 has occurred appropriately. With regard to the set of NgR1 mutants that did not bind to any of the ligands, an argument can be set forth that these mutants are simply misfolded. However, the continuous coiling structure of the LRR domain provides no *a priori* reasons to assume that mutations in the central region of the concave side of LRR domain would be more detrimental to the folding of the LRR domain than mutations in the periphery of the concave region.

4.6 Lrrtm1 and Lrrtm3 knock out mice as tools to pioneer *in vivo* studies on neuronal LRR membrane proteins

During this thesis work Lrrtm1 and Lrrtm3 knock out mice were generated. While more extensive characterization of these mouse lines is undergoing, a brief set of technical

observations verifying a successful gene targeting as well as the functionality of reporter genes is provided below.

4.6.1 Lrrtm1 and Lrrtm3 genes can be efficiently targeted

Successful targeting of Lrrtm1 and Lrrtm3 genes was verified by Southern blotting. Targeting strategy for the Lrrtm1 is shown in **Figure 10.** The absence of Lrrtm3 mRNA in then brain of an adult lrrtm3 knock out mouse is presented in **Figure 11**.

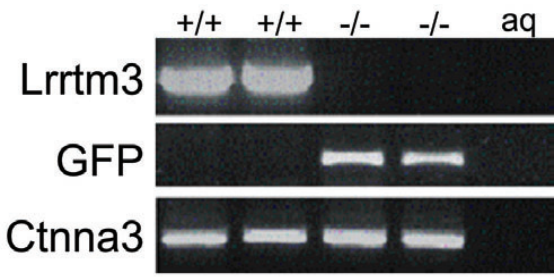

Figure 11. RT-PCR analysis of adult Lrrtm3 knock out (-/-) and wild-type (+/+) brain mRNA. *It demonstrates the complete removal of Lrrtm3 mRNA and the appearance of GFP reporter-gene mRNA transcript in the knock out mice. The mRNA expression of nested Ctnn3 gene is similar between the genotypes. Results are shown for two pairs of mice. aq, water control.*

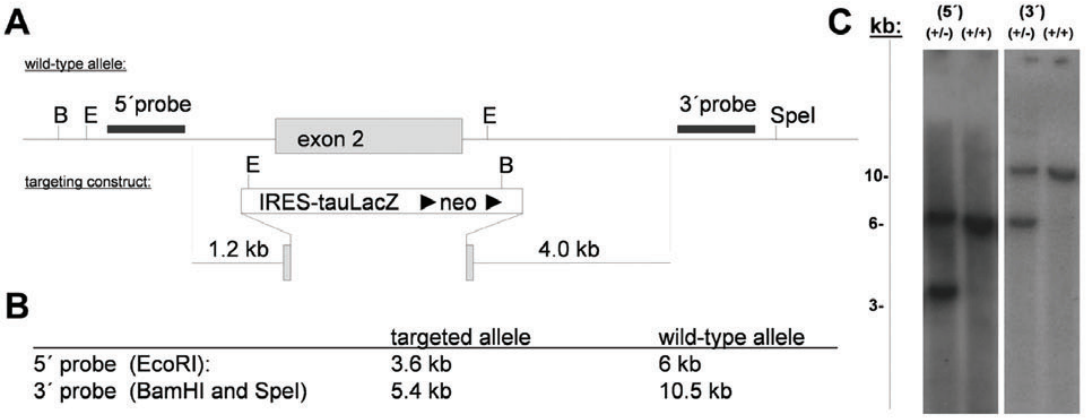

Figure 10. Targeting strategy and Southern blotting results for Lrrtm1 locus. *In (A) part of wild-type lrrtm1 locus as well as the elements of the replacing cassette are shown. The locations of the restriction enzyme cleavage sites used for Southern blotting analysis are also indicated. The expected lengths of Southern blotting fragments obtained with 5´ and 3´ probes, when digesting the ES cell genomic DNA with the indicated restriction enzymes, are shown in (B). The obtained Southern blotting results with correctly targeted cell clone (+/-) and wild-type (+/+) cell clone are shown in (C).*

4.6.2 tau-GFP and tau-LacZ reporter expression

Brains of adult Lrrtm3 or Lrrtm1 heterozygous mice were used to analyze the expression of the reporter genes encoding either tau-GFP (Lrrtm3 knock out) or tau-LacZ (Lrrtm1 knock out). The observed results not only identify the terminal fields of Lrrtm3 and Lrrtm1 expressing neurons, but also largely corroborate the earlier *in situ* hybridization results: *e.g.* for Lrrtm1, prominent mRNA expression was observed in the mitral cell layer of the olfactory bulb, and prominent LacZ activity was detected in the presumed innervation target, piriform cortex (data not shown). For Lrrtm3, prominent mRNA expression was detected in the granular cell layer in the cerebellum; as expected, abundant GFP immunoreactivity was observed in the molecular layer (**Figure 12**).

Thus the reporter genes can be used to map the neuronal connections of Lrrtm1 and Lrrtm3 expressing neurons in finer detail. While the LacZ reporter provided easy analysis, the GFP signal had to be visualized with anti-GFP antibodies (Chemicon) in combination with fluorescent or enzymatic secondary antibodies.

4.6.3 On the phenotype of Lrrtm3 knock out mice

Lrrtm1 and Lrrtm3 knock out mice are viable. Preliminary analysis of backcrossed Lrrtm3 knock out mice suggests that their performance in test requiring motor co-ordination (rotarod test) is compromised. This is in agreement with the extensive expression of Lrrtm3 mRNA in the cerebellum and basal ganglia. Among other tests performed Lrrtm3 knock out mice were found to be less immobile in a test measuring depression-like behavior (Matti Airaksinen, Vootele Voikar, Juha Laurén, unpublished).

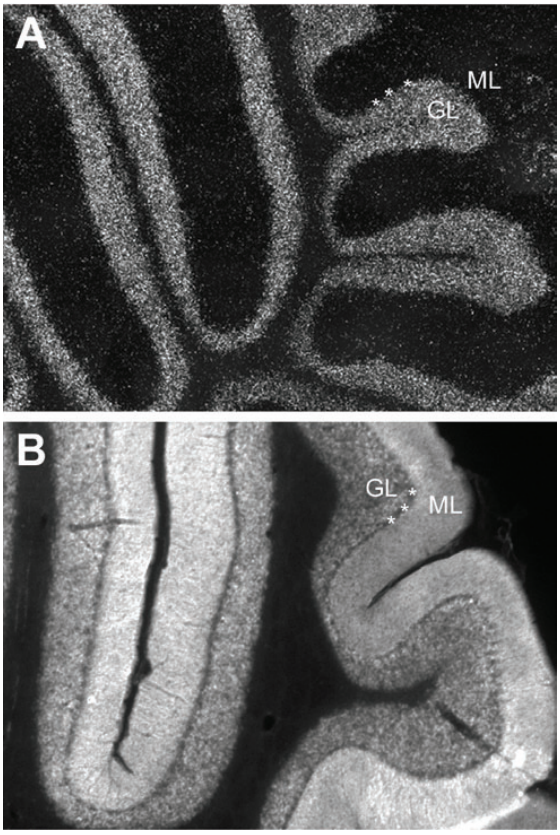

Figure 12. Lrrtm3 mRNA expression (A) and Lrrtm3 tau-GFP reporter expression in the adult mouse cerebellum. *ML, molecular layer; GL, granular layer; asterisks denote Purkinje cell layer. Lrrtm3 mRNA expression pattern in the cerebellum was first published in* ***(I)****; Fig. B kindly provided by Dr. Airaksinen.*

4.7 Association of LRRTM1 locus to schizophrenia and handedness

Dr. Clyde Francks, the director of our collaborative study on the genetics of handedness, found a strong association between a 70 kb-spanning haplotype in the proposed promoter region of LRRTM1 gene and a quantitative measure of human handedness (P=0.00002), when the haplotype was paternally inherited. LRRTM1 is located within the previously identified 2p12-q11 handedness gene candidate region. The predisposing haplotype (named as “2-2 haplotype”) is composed of two SNP and has a 9% frequency in the Caucasian population **(IV)**. Those who inherit the “2-2 haplotype”

were more likely to become left-handed than those who inherit some other haplotype.

By analyzing 1002 families of European origin affected with schizophrenia, Dr. Francks also found that the same LRRTM1 haplotype predisposes to schizophrenia (P=0.0014), when paternally inherited **(IV)**. Consistent with paternal association, in humans LRRTM1 may be an imprinted gene with a variable pattern of maternal down-regulation. In summary, our association data suggests that allelic variation upstream of LRRTM1 on the active paternal chromosome may affect LRRTM1 expression and play a role in the development of left-right brain asymmetry.

4.7.1 No association of LRRTM1 gene 5´ region to schizophrenia in Han Chinese families

After observing that the "2-2 haplotype" is associated with schizophrenia in Caucasian family samples, we wanted to test the possible association of the same haplotype with schizophrenia in Han Chinese schizophrenia samples. These biomaterials (DNA samples of 1151 individuals) and schizophrenia data were originally collected in three projects participating in the National Institute of Mental Health (NIMH) Schizophrenia Genetics Initiative.

The scatter diagram classifying the PCR products, and hence the genotypes, demonstrates clearly distinguishable SNP base calling results in nearly all cases **(Figure 13)**. The sequences of the primers use in the genotyping are shown in **Table 7**. Mendelian inheritance of the observed genotypes was verified, and families with non-Mendelian inheritance pattern were removed from the analysis. In total, one family was removed from the analysis of rs723524 SNP and five from the analysis of rs1446109 SNP. These inconsistencies were from errors in genotyping, or errors in identifying the biological father of the offspring. The number of families removed from the analysis has a negligible effect on the statistical analysis.

For the analysis, a transmission disequilibrium test (TDT) was used. This test looks at all heterozygous parents and scores which alleles are transmitted to affected offspring. A deviation from 50-50 transmission of the alleles, summed across all families, would be indicative that the SNP or haplotype is associated with the disease – either causally related to the disease, or in linkage disequilibrium with a nearby causal variant. As we were testing a hypothesis related to an imprinted gene, we tested paternal and maternal transmission separately. The statistical analysis was mostly performed by Dr. Clyde Francks.

We did not find evidence for paternal over-transmission of the risk haplotype to schizophrenia patients or any other association of schizophrenia to this locus in Han Chinese population **(Tables 8-10)**. This suggests that the LRRTM1 "2-2 haplotype", that is associated with schizophrenia in Caucasian samples, is not associated with schizophrenia in Han Chinese population.

It is plausible that ethnic groups of different genetic composition have, due to population bottleneck effects, unique collections of different gene variants that predispose to schizophrenia. Alternatively, the LRRTM1 locus might be associated with schizophrenia in Han Chinese population, but the predisposing haplotype could be determined by different SNPs. To clarify this matter, more extensive genotyping should be performed to define the main genetic variations in this locus in Han Chinese population, and to determine if some other haplotype is associated to schizophrenia.

In the Caucasian population, the rare alleles of SNPs rs1446109 and rs723524 have each ~10% frequency. In the Han Chinese population the rare alleles had ~25% and ~35% frequency, respectively. This pattern is consistent with HapMap data; allele frequency differences of this magnitude are common between populations (HapMap Consortium 2005).

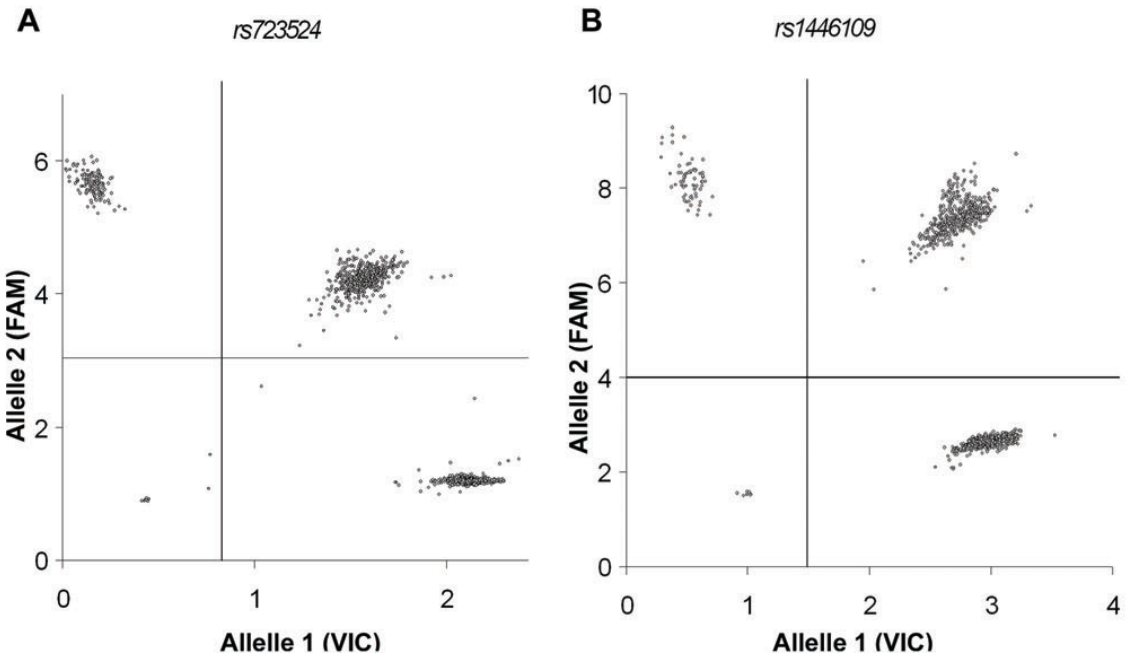

Figure 13. Scatter diagram classification of the PCR products. *The fluorescent dyes detected are indicated on the X and Y-axis. The cut-off limits used are depicted by horizontal and vertical lines. For example, for rs723525 samples in the upper left quadrant are homozygous for allele 2, samples in the upper right quadrant are heterozygous, and samples in the lower right quadrant are homozygous for allele 1. The PCR-reaction did not produce a detectable amplicon for the samples in the lower left quadrant.*

SNP ref #	Assay context sequence
rs723524	TACATGACTTGATCATGTGCAGAAT**[G/T]**ATAGGGAAATATATTTCCAACACAG
rs1446109	TACATAATGCCAAATTGTCATCCTG**[A/G]**AAGATTTCACACTTTTCATCCTCAA

Table 7. Sequences of the primers used in the genotyping. *The primers labeled with VIC and FAM differed in one nucleotide (in brackets), and hence specifically amplify only one of the SNP variants.*

			Paternal			Maternal			Combined		
	N	%	TR	NT	Chi^2	TR	NT	Chi^2	TR	NT	Chi^2
rs1446109.1	799	74.5	95	84	0.68	73	78	0.17	210	204	0.09
rs1446109.2	273	25.5	84	95	0.68	78	73	0.17	204	210	0.09

Table 8. Transmission data for locus rs1446109. *N, number of individuals with allele ".1" [=A] or ".2" [=G]. %, percentage of individuals with allele ".1" or ".2". TR, number of times allele is transmitted to affected individual. NT, number of times allele is not transmitted to affected individual.* Chi^2 *is a statistical measure reporting the likelihood that the observed distribution differs from what is expected under random inheritance model. Analysis was performed for paternal, maternal and overall inheritance. For example allele ".2" of rs1446109 has 25.5% frequency and it was transmitted by heterozygous fathers to affected offspring 84 times in the sample, and not transmitted 95 times. This is not significantly different from what could be expected by chance.*

			Paternal			Maternal			Combined		
	N	%	TR	NT	Chi^2	TR	NT	Chi^2	TR	NT	Chi^2
rs723524.1	692	64.9	89	81	0.38	91	89	0.02	233	223	0.22
rs723524.2	374	35.1	81	89	0.38	89	91	0.02	223	233	0.22

Table 9. Transmission data for locus rs723524. Allele ".1" is [G] and allele ".2" is [T].

			Paternal			Maternal			Combined		
	N	%	TR	NT	Chi^2	TR	NT	Chi^2	TR	NT	Chi^2
Haplotype 1	582	59.1	106	87	1.87	93	98	0.13	213	199	0.48
Haplotype 2	177	18.0	65	78	1.18	66	62	0.12	141	150	0.28
Haplotype 3	158	16.1	54	57	0.08	59	64	0.20	117	125	0.26
Haplotype 4	67	6.8	31	34	0.14	28	22	0.72	59	56	0.08

Table 10. Transmission data for haplotypes formed by alleles extending from rs1446109 to rs723524 .

5 CONCLUDING REMARKS

This study characterizes evolutionary origins and expression pattern of six previously uncharacterized genes: LRRTM1-4 and NGRL2-3. These genes encode proteins belonging to the neuronal LRR membrane protein family. LRRTM and NGRL mRNAs are prominently expressed in the human and mouse brain, and they possess partially non-overlapping expression patterns. When overexpressed, all NgR family members are to a significant degree transported to the plasma membrane. Interestingly, at least LRRTM1 is localized intracellularly in all cell types tested when overexpressed.

While this thesis work was being completed, a whole collection of other studies addressing the structure and functions of other neuronal LRR membrane proteins was also published. The emerging picture is that neuronal LRR membrane proteins play a role in neurite growth regulation, synapse formation, and in the regulation of growth factor signaling. This alone justifies future detailed studies on the function of these genes. The knock out mouse lines that were developed as a part of this thesis work will undoubtedly be useful.

This study clarifies several important aspects on ligand binding to NgR1, and defines new high-affinity molecular interactions: I found that three separate segments of Nogo-A interact with NgR1, and that RTN2-66 and RTN3-66 interact with NgR1 as well. Since I observed that all RTNs are most prominently expressed in the neurons, 66 amino acid loops of RTNs 2, 3, and 4 could interact with NgR1 *in cis* in neurons. However, RTN3 mRNA splice variant encoding protein with the RHD was also detected in the glial cells of the adult mouse spinal cord. Thus RTN3-66 could have a role in myelin inhibition of neurite growth.

The extensive NgR1 mutagenesis study defined a central region on the concave side of NgR1 that is critical for the binding of Nogo-66, MAG, and OMgp. This compact core binding region might be amenable to pharmacological interventions.

With regard to LRRTM1, our association data suggests that allelic variation upstream of LRRTM1 may affect the gene's expression on the active paternally inherited chromosome. This altered gene expression might be relevant to the formation of brain asymmetry. LRRTM1 is the first identified potential genetic influence on human handedness, and the second putative gene involved in human brain asymmetry. How LRRTM1 might affect brain development at certain locations and time points remains as an important open question.

Since schizophrenia is associated to left-handedness, we studied the association of LRRTM1 “2-2 haplotype” with schizophrenia. We found that LRRTM1 “2-2 haplotype” is associated with schizophrenia in certain Caucasian populations, but not among Han Chinese. Schizophrenia, like handedness and brain asymmetry, is an etiologically complex trait with many genetic and environmental influences.

6 FUTURE DIRECTIONS

The overall role of Nogo-66 Receptor is not dramatically clearer today than what it was at the time of its discovery in 2001, when it was postulated that “Disruption of the interaction between Nogo-66 and its receptor provides the potential for enhanced recovery after human CNS injury” (Fournier *et al.* 2001). *In vitro* experiments have revealed a growing list of proteins or protein fragments that bind to NgR1 with nanomolar-level affinity. Currently, proteins or protein fragments known to bind to NgR1 with very high affinity are Nogo-66, RTN2-66, RTN3-66, Nogo-Y4C, Nogo-C39, MAG, OMgp, p75, Taj/TROY, Lingo1, Abeta1-42, FGF-2 (Dr. Roman Giger, Society for Neuroscience meeting abstract, 2006), and three currently unpublished proteins identified in Dr. Strittmatter’s laboratory. The full biological importance of all these interactions should be evaluated in the future experiments.

Technical challenges in efficiently introducing and down-regulating genes in primary neuron cultures as well as those in generating highly specific and potent preparations of myelin inhibitors of axonal growth are significant. However, with time a more informed consensus regarding the role and importance of Nogo-66, NgR1, and the proposed co-receptors in myelin inhibition of neurite growth, will likely emerge.

More research is needed to address the physiological role of the high affinity interaction between MAG and OMgp that I described in this work.

Future studies on the roles of LRRTM1 in mammalian brain development may reveal new insights into one of the major uncharacterized processes in developmental biology, the establishment of the left-right brain axis. Defects in LRRTM1 function could potentially lead to maldevelopment of left-right asymmetry. Thus variation in the LRRTM1 locus might have a role in not only schizophrenia but also in other neuropsychiatric disorders.

The Lrrtm1 knock out mice that I made as a part of this thesis work can be used to i) study *in vivo* functions of LRRTM1; ii) study the mechanisms that lead to left-right brain asymmetry; and iii) gain insight into the etiology of schizophrenia by analyzing the possible schizophrenia-associated behavioral disturbances in these mice.

First, the expression of Lrrtm1 in the developing mouse CNS should be analyzed in detail by utilizing the tau-LacZ reporter gene.

Second, whether Lrrtm1 knock out mice exhibit behavioral disturbances or endophenotypes that are potentially relevant to signs and symptoms of schizophrenia (such as psychomotor agitation, sensitivity to psychotomimetic drugs, social withdrawal, disturbed working memory and spatial learning, deficits in attention/sensorimotor gating; for review, see (Powell and Miyakawa 2006)) should be examined in detail.

Third, to assess whether Lrrtm1 knock out mice exhibit abnormally strongly or weakly lateralized paw preference, the strength of lateralization of paw-preference (i.e. how consistently using the same forepaw) should be studied using established pellet-reaching assays (Collins 1968).

Finally, to mechanistically understand how variation in the LRRTM1 locus might partially determine handedness in humans, detailed studies on CNS development in the Lrrtm1 knock out mice should be performed.

Left-right brain asymmetry could result from differential timing of key developmental events in contrasting hemispheric regions. In a simplified form, brain asymmetry could be caused by a delay in mitotic exit of neuronal progenitor cells in either side of the brain. This would allow more neurons to emerge in that hemispheric structure. Alternatively, it is also plausible that differential timing in cortical maturation facilitates certain regions of the left or right side of the cerebral cortex to form functionally preferred thalamocortical connections, which would impose different functional identities on the otherwise equivalent cortical regions in the left and right hemispheres.

To determine the possible role of Lrrtm1 in controlling neuronal mitotic exit, migration and size of key brain structures (most importantly the cerebral cortex and its layers) in Lrrtm1 knock out mice should be determined and compared to wild-type control mice. This analysis can be complemented by determining neuronal cell density. The quantity and anatomical destiny of neurons born during given developmental time points should be assessed by 5-bromo-2´-deoxyuridine (BrdU) labeling. The neuronal morphology in Lrrtm1 knock out brain could be studied *in situ e.g.* by crossing Lrrtm1 knock out mice with Thy-1-EGFP transgenic line, which expresses GFP in a subset of neurons thereby enabling detailed studies on axonal, dendritic and spine morphology by confocal microscopy.

A recent study found that LRRTM3 promotes processing of amyloid precursor protein (APP) by BACE1 (Majercak *et al.* 2006). This finding is especially significant as it came from a systematic RNAi screening of 15 200 genes for their role in Aβ42 secretion. Majercak *et al.* also noted that LRRTM3 maps to a chromosome 10 locus linked to late-onset Alzheimer's disease and elevated plasma Aβ42 levels in humans. However, the study by Majercak *et al.* was based exclusively on *in vitro* experiments. To assess the gene's proposed role in Alzheimer's disease pathogenesis, *in vivo* experiments using Lrrtm3 knock out mice are now much looked after.

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